PREPUPAL PARASITOID Mastrus ridibundus EAVESDROP ON PHEROMONAL COMMUNICATION OF COCOON-SPINNING Cydia pomonella LARVAE

by

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ABSTRACT

As shown in a recent study, cocoon-spinning larvae of the codling moth, *Cydia pomonella* L. (Lepidoptera: Olethreutidae) employ a pheromone that attracts or arrests pupation site seeking conspecific larvae. Such intraspecific communication signals are important cues for illicit receivers such as parasitoids to exploit. I tested the hypothesis that the specialist prepupal *C. pomonella* parasitoid *Mastrus ridibundus* Gravenhorst (Hymenoptera: Ichneumonidae) exploit the larval aggregation pheromone to locate host prepupae. In laboratory olfactometer experiments, female *M. ridibundus* were attracted to 3-day-old cocoons containing *C. pomonella* larvae or prepupae. Older cocoons containing *C. pomonella* pupae, or larvae and prepupae excised from cocoons, were not attractive. In coupled gas chromatographic-electroantennographic detection (GC-EAD) analyses of bioactive Porapak Q extract of cocoon-derived airborne semiochemicals, ten compounds elicited responses from the antennae of female *M. ridibundus*. Comparative GC-mass spectrometry of authentic standards and cocoon-derived volatiles determined that compounds were 3-carene, myrcene, heptanal, octanal, nonanal, decanal, (E)-2-octenal, (9Z)-2-nonenal, sulcatone, and geranylacetone. A synthetic 11-component blend consisting of these ten EAD-active compounds plus EAD-inactive (+)-limonene (the most abundant cocoon-derived volatile) was as effective as Porapak Q cocoon extract in attracting both female *M. ridibundus* and *C. pomonella* larvae seeking pupation sites. Only three components [myrcene, (E)-2-nonenal, (+)-limonene] could be deleted from the 11-component blend without diminishing its attractiveness for *M. ridibundus* which
underlines the complexity of information conveyance during host foraging. *Mastrus ridibundus* obviously eavesdrop on the pheromonal communication signals of *C. pomonella* larvae that reliably indicate host presence. Whether the larval aggregation pheromone is as complex as the semiochemical blend attracting *M. ridibundus* is currently under investigation.
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GLOSSARY

**Communication.** The process in which one individual uses specially designed signals or displays to modify the behaviour of another (Krebs and Davies 1998).

**Eavesdrop.** The interception and exploitation by a parasitoid of semiochemical signals from its host (Stowe et al. 1995).

**Fresh cocoon.** Cocoon material collected within three days from the onset of cocoon-spinning activity by larvae.

**Gregarious parasitoid.** A parasitoid species with one or more individuals developing in or on the same host (Godfray 1994).

**Illicit receiver.** A parasitoid which exploits the communication signals produced by other organisms to find resources (Haynes and Yeargan 1998).

**Naïve parasitoid.** A parasitoid that has not had previous experience with a particular test stimulus.

**Non-responding insects.** Experimental insects which did not show a positive response to either the treatment or the control stimulus.

**Parasitoid.** The larva of an arthropod that feeds exclusively on the body of another arthropod, its host, eventually killing it (Godfray 1994).
1.0. INTRODUCTION

Host location by hymenopterous parasitoids has been reported to proceed in four steps: location of host habitat, location of host, acceptance of host, and determination of host suitability (Doutt 1959; Vinson and Iwantsch 1980; Vinson 1976, 1984a,b, 1998). In many parasitoids, host habitat location seems mediated by semiochemicals from the food of the host (Vinson 1981; Vet et al. 1991; Vet and Dicke 1992), whereas host location often is mediated by semiochemicals directly from the host insect (Stowe et al. 1995, Haynes and Yeargan 1999). In many tritrophic communication systems, parasitoids respond to qualitative and quantitative changes in semiochemicals emanating from food plants fed on by host larvae (Turlings et al. 1991; Tumlinson et al. 1993; Alborn et al. 1997; De Moraes et al. 1998; Mattiacci et al. 1999). To locate their hosts, parasitoids exploit diverse chemical cues that are produced directly or indirectly by potential hosts and reliably indicate their presence (Vet et al. 1995). These semiochemicals emanate from the host itself (moth scales; DeLury et al. 1999) or its metabolites (excrement, silk; Lewis and Jones 1971; Weseloh 1976; Mattiacci et al. 1999).

In many systems, parasitoids are attracted to their host by semiochemicals emitted by their future “victims” (Stowe et al. 1995). Host foraging parasitoids are faced with a reliability-detectability problem. Semiochemicals from the first trophic level are highly detectable and direct parasitoids to host habitat but are poor indicators of host presence. Semiochemicals from the host itself, in contrast, are reliable indicators of host presence, but are scarcely detectable to parasitoids because host insects express a low
semiochemical profile (Vet et al. 1991; Vet and Dicke 1992; Stowe et al. 1995).
Parasitoids that learn to associate these highly detectable host-habitat derived
semiochemicals with highly reliable host-produced semiochemicals enhance their
foraging effectiveness and increase their Darwinian fitness (Vet and Dicke 1992;
Hoffmeister and Roitberg 1997a,b; Geervliet et al. 1998; Hoffmeister et al. 2000).

While maintaining a low semiochemical profile is generally advantageous to
hosts, they cannot completely avoid emitting semiochemicals such as those that may
serve as intraspecific signals to attract mates, mark oviposition sites, or defend territories
(Stowe et al. 1995, Hedlund et al. 1996). As such, these intraspecific communication
signals may become important cues for illicit receivers to exploit (Stowe et al. 1995;
Haynes and Yeargan 1999).

*Mastrus ridibundus* (Gravenhorst) (Hymenoptera: Ichneumonidae) is an
introduced gregarious ectoparasitoid specialising on late instar/prepupal codling moth,
*Cydia pomonella* L. (Lepidoptera: Olethreutidae), an exotic pest of apples and other
pome fruits in North America (Pedigo 1999; Bezemer and Mills 2001). In its native
Kazakhstan, *M. ridibundus* may parasitize ≥ 60% of overwintering *C. pomonella*
prepupae.

Observations (Tom Unruh, USDA-Agricultural Research Station, Wapato,
Washington; personal communication) suggested that female *M. ridibundus* are attracted
to semiochemicals emanating from cocoon-spinning *C. pomonella* larvae and prepupae.
Moreover, Duthie et al. (2003) provided evidence that cocoon-spinning *C. pomonella*
larvae produce an aggregation pheromone that mediates aggregation/arrestment of
pupation site seeking larvae in a microlocation. This aggregation pheromone is produced
and detectable, respectively, in the cocoon-spinning larval and prepupal stage but not in
the pupal stage (Duthie et al. 2003). Conceivably, *M. ridibundus* may eavesdrop on the
intraspecific communication of *C. pomonella* larvae by exploiting the larval aggregation
pheromone as a reliable and detectable host derived semiochemical.

My objectives were: 1) to test the hypothesis that semiochemicals from cocoon-
spinning *C. pomonella* larvae attract host foraging female *M. ridibundus*; 2) to determine
the semiochemical source and longevity; 3) to identify the semiochemicals; and 4) to
investigate whether *M. ridibundus* eavesdrop on pheromonal communication by cocoon-
spinning *C. pomonella* larvae.
2.0. LIFE HISTORIES

2.1. Cydia pomonella

_Cydia pomonella_ (Figure 1a) peak flight occurs just prior to and after sunset and, under favourable conditions, may last for up to 2 hr (Dolstad 1985; Howell _et al._ 1990). Optimal temperatures for flight range from 12-33°C, above and below which flight activity ceases (Dolstad 1985). At dusk, females call potential mates by releasing a long-range sex pheromone blend comprising (E,E)-8,10-dodecadienol (Roelofs _et al._ 1971), the primary component, and secondary components (E,Z)-8,10-dodecadienol, (E)-9-dodecanol, dodecanol, and tetradecanol (El-Sayed 1999). At close range, females may rely on visual stimuli in accepting a mate (Weissling and Knight 1994). Gravid and mated females oviposit eggs on or near fruit during sunset and may deposit an epideictic pheromone after oviposition (Thiery _et al._ 1995).

Mated females deposit 30-50 eggs (Geier 1963; Pedigo 1999; Unruh and Lacey 2000) singly directly on the apple, pear, or walnut fruit or near the fruit on host foliage (Summerland and Steiner 1943; Thiery _et al._ 1995; Landolt _et al._ 1999). The freshly laid egg is white and convex, and appressed closed to the substrate (Dolstad 1985). As the embryo develops, the egg darkens following melanization and a red ring surrounds the developing insect. Finally, 1-2 days prior to hatching, the head capsule of the larva becomes visible through the egg as a black spot (Dolstad 1985). Neonate larvae hatch 5-15 days after oviposition and commence foraging for fruit within a few hours (Pedigo 1999).
Figure 1  Life cycle of (a) the codling moth, *Cydia pomonella*, and (b) its prepupal parasitoid, *Mastrus ridibundus*. 
Adult: Lives 14 to 21 days

Pupa (inside cocoon): 8 to 30 days

Egg: Hatches after 5 to 15 days

Larva (5 instars): Feeds for 21 to 35 days.

Pupa (inside host cocoon): 10 to 14 days

Egg: Hatches after 2 to 5 days

Larva: Feeding on host larva for 4 to 7 days
Larvae complete five instars before pupating. The white first instar larva is ca. 1.5 mm in length with a black head capsule, whereas the mature fifth instar is ca. 20 mm in length with a brown head capsule and cervical shield, and a pinkish body (Dolstad 1985).

Neonate larvae rely partially on semiochemicals from apple skin to guide them to the fruit within a few hours post-hatching (Dolstad 1985; Landolt et al. 1999). Once an apple fruit is located, larvae burrow in through the calyx leaving a sting mark, and continue to feed on the flesh of the fruit until they reach the core within which they attack the seeds (Dolstad 1985; Unruh and Lacey 2000, Higbee et al. 2001). The number of larvae per fruit is limited due to aggressive interactions between third instar larvae. Mature fifth instars burrow out of the apple, usually causing a second sting mark, and seek cryptic and protective microhabitats, such as cracks or crevices within the tree’s bark, ground litter, or in other shelters, where they will spin a cocoon and pupate in aggregations (Geier 1963; Unruh and Lacey 2000; Duthie et al. 2003). The time from larval hatching to pupation takes 14-35 days (Geier 1963). Pupae are dark brown in colour, and the pupal period lasts 8-30 days (DeLury 1998).

_Cydia pomonella_ are protandrous. Adult males eclose up to 2 days prior to females (Duthie et al. 2003). The moths are 6-7 mm long with an average wingspan of 19 mm (Dolstad 1985). They are mostly grey with copper coloured spots lying distally on the forewing. A buff coloured variant is light brown in colour (Dolstad 1985; Pedigo 1999). At rest, the wings are held roof-like over the body (Dolstad 1985). Adult moths live for 14-21 days in the wild (Pedigo 1999).
2.2. *Mastrus ridibundus*

*Mastrus ridibundus* (Figure 1b) was originally collected in Kazakhstan in 1994 and released into North America in 1995 (Unruh 1997; Kuhlmann and Mills 1999). *Mastrus ridibundus* is a gregarious ectoparasitoid specialising on cocooning late instar/prepupal *C. pomonella* larvae. Female *M. ridibundus* primarily attack *C. pomonella*, and only rarely attack congeneric hosts (Kuhlmann and Mills 1999) or *Grapholita prunivora* (Walsh) (Dhalsten 1994), which is also a minor pest in apple orchards.

Female *M. ridibundus* deposit 1-10 eggs on the integument of the host insect after stinging and paralyzing it (Abdullaev 1974; Makarov 1983; Dhalsten 1994). Parasitoid larvae hatch 1-3 days after oviposition (personal observation) and continue for 4-7 days to feed on the *C. pomonella* host until only the host’s head capsule and integument remains (personal observation). Final fifth instar *M. ridibundus* proceed to spin their cocoons within the cocoon of their *C. pomonella* host before pupating. Depending upon whether host larvae are overwintering (diapausing) or not, *M. ridibundus* will spin light-dark brown cocoons and thin white cocoons, respectively (Abdullaev 1974; Dhalsten 1994). Non-diapausing adults eclose within 10-14 days after pupating, whereas diapausing adults eclose the following spring. The sex ratio of adult parasitoids is typically 1:1 (Tom Unruh, personal communication).

Male *M. ridibundus* eclose 2-3 days before females. Adults are 5-6 mm in length with a black head and thorax, and orange legs and abdomen. The antennae are ca. 3 mm long, and the wings are transparent and shaded light brown/grey. Females with their protruding 3 mm long ovipositor can easily be distinguished from males. Although there
is much variation in adult size, most likely reflecting the number of larvae per host, (Horstmann 1990), adult females generally tended to be larger than adult males (personal observation).

Females and males live for 14-21 days at 22-24°C (Isankalova 1972; Abdullaev 1974) and may mate shortly after emergence from cocoons. Females do not exhibit any kind of calling behaviour prior to mating (personal observation). *Mastrus ridibundus* are arrhenotokous; impaternate males develop from unfertilized eggs, whereas females develop from fertilized eggs.
3.0. METHODS AND MATERIALS

3.1. Experimental Insects

Cydia pomonella. Larvae reared on artificial diet were shipped from the Sterile Insect Release Program in Osoyoos, British Columbia, Canada. The colony has been maintained since 1991 with periodic introductions of wild individuals, the last introduction occurring in 2001. Trays containing ca. 1000 larvae were kept in a glass aquarium (60 × 31 × 31 cm) and stored at 15°C under a photoregime of 16L:8D. To generate a test stimulus for bioassay experiments, five final instars were removed from the diet and allowed to cocoon on a corrugated cardboard strip (2.5 × 2.5 cm) (0.46 m × 76 m single face corrugated cardboard; Shippers Supply Inc., British Columbia, Canada) placed in a 35 mm plastic Petri dish. If the test stimulus consisted of cocoons, larvae were excised from their cocoons ~1.5 hr prior to bioassays.

Mastrus ridibundus. Cydia pomonella hosts parasitized with M. ridibundus were shipped in corrugated cardboard rolls from the USDA-Agricultural Research Station, Wapato, Washington, USA. The colony has been maintained since 1996. Shipments included both diapausing and non-diapausing M. ridibundus.

Rolls with non-diapausing M. ridibundus were kept in a partially meshed Plexiglass cage (40 × 30 × 30 cm) maintained at 20-23°C, 30-50% RH, and a 16L:8D photoperiod. Adult parasitoids emerged from the rolls during a 5-d period, and were sustained with a 10% honey-water solution and a water-soaked cotton wick ad libitum. All insects used in bioassays were allowed a minimum two-day pre-oviposition period (Tom Unruh; personal communication) and were between 3-14 days old at the time of the
experiment. Rolls with diapausing *M. ridibundus* were stored at 3°C in complete darkness. Diapause was broken by exposing insects to a 16L:8D photoperiod and 20-23°C. Parasitoids began to emerge after 17-20 d.

Female parasitoids were transferred to a small wooden cage (13 × 18 × 18 cm) with a sliding Plexiglass front and a meshed back one day prior to experiments, and transferred singly to plastic cups (4 × 4 cm) 1 hr prior to experiments to allow acclimation. Naïve parasitoids were used in all bioassay experiments and each insect was tested only once. Although mating status was not confirmed, females spent three or more days with males and were very likely mated (personal observation).

### 3.2. Acquisition of Volatiles

To solvent-extract *C. pomonella* cocoons, 460 late instar larvae were allowed to cocoon in a Sparkleen™ washed glass aquarium (60 × 31 × 31 cm) for three days. Three hr before bioassays, larvae were excised from cocoons, and cocoons removed from the aquarium and extracted in methanol (MeOH). Twenty cocoon extract equivalents (20 CEE = 230 µl of methanol extract from 20 *C. pomonella* cocoons) were tested per replicate.

To collect airborne volatiles from cocoon-spinning larvae, three-hundred 5th instar larvae were removed from the diet, placed in a cylindrical Pyrex® glass chamber (15.5 ID × 20 cm high), and aerated for ~72 hr. A water aspirator drew charcoal-filtered air at 2 litres/min through the chamber and a downstream glass column (1400 × 10.1 mm ID) filled with Porapak Q (50-80 mesh, Waters Associates, Inc., Milford, Massachusetts 01757). Volatiles were eluted from the Porapak Q with 3 ml of solvent [redistilled pentane:ether (95:5)]. The eluent was concentrated to 1 ml adjusting the volatile extract
so that 1 µl was equivalent to 10 cocoon-spinning larvae hour equivalents (10 CSLHE = volatiles released from 10 cocoon-spinning *C. pomonella* larvae during 1 hr).

### 3.3. Analyses of Volatiles

Aliquots of 20 CSLHE of Porapak Q extract were subjected to analysis by coupled gas chromatographic-electroantennographic detection (GC-EAD) (Arn *et al.* 1975) employing a Hewlett Packard (HP) 5890A gas chromatograph equipped with a fused silica column (30 m × 0.25 or 0.32 mm ID) coated with DB-5, DB-210, or DB-23 (J & W Scientific, Folsom, California, USA). For GC-EAD recordings, the proximal end of a severed female *M. ridibundus* antenna was placed into the opening of a glass capillary electrode (1.0 × 0.58 × 100 mm) (A-M Systems, Inc., Carlsborg, Washington, USA) filled with saline solution. The distal end with its tip removed by spring microscissors (Fine Science Tools Inc., North Vancouver, British Columbia, Canada) was placed into the recording capillary electrode (Gries *et al.* 2002).

Full scan electron impact (EI) mass spectra of EAD-active compounds were obtained by GC-mass spectrometry (MS), using a Varian Saturn 2000 Ion Trap GC-MS, and a HP 5985B GC-MS, respectively, each fitted with the DB-210 or DB-5 column referred to above.

### 3.4. Olfactometer Bioassays

#### 3.4.1. Y-tube Olfactometer Bioassays

Anemotactic responses of naïve female *M. ridibundus* to test stimuli were bioassayed in a vertically oriented Y-tube Pyrex® glass olfactometer (ID 23 mm; stem 25 cm; arms 20 cm at a 120° angle) at 20-25°C and 40-70% RH. Air drawn through the apparatus at ~1.2 L/min with a water aspirator carried volatiles from each arm of the Y-
tube olfactometer toward upwind traveling parasitoids released individually into the stem. For each replicate, the position of the odour source was randomly assigned to Y-tube arms. A parasitoid that penetrated ≥10 cm into one arm within 15 min was classified as a responder. All non-responding insects were excluded from statistical analyses. Each replicate employed a new odour source, Y-tube, and parasitoid. During bioassays, a single light source composed of one fluorescent “daylight” tube (F40D Hb58; Osram Sylvania Ltd., Ontario, Canada) and one “wide spectrum” grow light (F40GRO WS6 H568; Osram Sylvania Ltd., Ontario, Canada) was centered ~20 cm above the olfactometer. Y-tubes were washed between replicates with Sparkleen™ and dried at 125°C for ≥ 30 min.

Experiments 1-3 (Table 1) tested potential sources of semiochemicals (larvae/prepupae, cocoon) derived from *C. pomonella* working under the hypothesis that *M. ridibundus* females are attracted to semiochemicals emanating from *C. pomonella* cocoons. Taking into account that semiochemical attractiveness resided with the cocoons, experiments 4-9 (Table 1) explored whether the age of cocoons affects their semiochemical attractiveness hypothesizing that aged cocoons will be less attractive than fresh cocoons due to cessation of production and degradation/dissipation of the semiochemical respectively. Experiments 10-11 (Table 1) then tested whether semiochemicals could be solvent extracted from cocoons (experiment 10), whether aging of extracts affected their attractiveness (experiment 11) and whether solvent washed cocoons still maintained attractiveness (experiment 12). Experiments 13-15 (Table 1) explored whether Porapak Q extracts of airborne cocoon-derived semiochemicals are attractive (experiment 13), and whether dose (experiment 14) or age (experiment 15) of
extracts affected their attractiveness. Considering the strong attraction of *M. ridibundus* to Porapak Q extract of cocoon semiochemicals (experiment 13), experiments 16-21 (Table 1) explored which semiochemicals were essential for blend attractiveness by testing specific blends lacking classes of organic chemicals (saturated aldehydes, unsaturated aldehydes, ketones, or monoterpenes). Follow-up experiments 22-32 (Table 1) determined which individual compounds were essential components of the semiochemical blend. Experiments 33-34 (Table 1) tested the 8-component semiochemical blend lacking all non-essential components [(E)-2-nonenal, myrcene, and (+)-limonene] *versus* Porapak Q cocoon extracts and *versus* the 11-component semiochemical blend respectively.

### 3.4.2. Petri-dish Pitfall Olfactometer Bioassay

Responses of pupation site seeking 5th instar *C. pomonella* larvae were tested in binary choice Petri dish pitfall olfactometers (Duthie *et al.* 2003). Test stimuli were randomly assigned to one of two 4 mL "pitfall" vials (Table 1) equipped with modified Eppendorf tubes to prevent contact of the experimental insects with a test stimulus. For each replicate, one fifth-instar larva was placed in the centre of the olfactometer, and its pupation site was recorded 18-24 hr later. All non-responding insects were excluded from statistical analyses. Olfactometers were kept at 21-26°C in complete darkness.

Experiment 35 (Table 1) tested attractiveness/arrestment of 200 CSLHE (see above) of a complete blend of synthetic semiochemicals *versus* that of a pentane control to determine if the same semiochemicals utilised by *M. ridibundus* females in host location also serve as an aggregation pheromone for *C. pomonella* larvae seeking pupation sites.
Table 1  Details on experimental insects and stimuli tested in olfactometer bioassays.
<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>♀ <em>M. ridibundus</em> (n)</th>
<th>Y-tube olfactometer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>36</td>
<td>Cocoon</td>
</tr>
<tr>
<td>2</td>
<td>29</td>
<td>Cocoon + cardboard (CB*)</td>
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<td>3</td>
<td>35</td>
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<td>32</td>
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<td>5</td>
<td>36</td>
<td>3-d-old cocoon + larva/ prepupa + CB</td>
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<td>6</td>
<td>34</td>
<td>7-d-old cocoon + pupa + CB</td>
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<td>7</td>
<td>31</td>
<td>13-d-old cocoon + pupa + CB</td>
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<td>31</td>
<td>3-d-old cocoon + larva/ prepupa + CB</td>
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<td>9</td>
<td>32</td>
<td>3-d-old cocoon + larva/ prepupa + CB</td>
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<tr>
<td>10</td>
<td>18</td>
<td>MeOH cocoon extract at 20 CEE (1-hr-old)</td>
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<tr>
<td>11</td>
<td>18</td>
<td>MeOH cocoon extract at 20 CEE (8-d-old)</td>
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<tr>
<td>12</td>
<td>17</td>
<td>20 MeOH washed cocoons</td>
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<tr>
<td>13</td>
<td>19</td>
<td>Porapak Q extract (1 CSLHE, 5-d-old)</td>
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<td>14</td>
<td>19</td>
<td>Porapak Q extract (1 CSLHE, 17-d-old)</td>
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<td>15</td>
<td>13</td>
<td>Porapak Q extract (50 CSLHE)</td>
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<td>16</td>
<td>18</td>
<td>Synthetic blend (SB^e)</td>
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<tr>
<td>Experiment No.</td>
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<td>Y-tube olfactometer</td>
</tr>
<tr>
<td>---------------</td>
<td>--------------------------</td>
<td>---------------------</td>
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<tr>
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<td>SB minus unsaturated aldehydes</td>
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<tr>
<td>19</td>
<td>17</td>
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<tr>
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<td>17</td>
<td>SB minus ketones</td>
</tr>
<tr>
<td>21</td>
<td>17</td>
<td>SB minus monoterpenes</td>
</tr>
<tr>
<td>22</td>
<td>17</td>
<td>SB minus decanal</td>
</tr>
<tr>
<td>23</td>
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<td>18</td>
<td>SB minus heptanal</td>
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<td>19</td>
<td>SB minus (E)-2-nonenal</td>
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<td>SB minus (E)-2-octenal</td>
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<td>28</td>
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<td>32</td>
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<td>33</td>
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</tr>
<tr>
<td>34</td>
<td>19</td>
<td>SB</td>
</tr>
<tr>
<td>Experiment No.</td>
<td>C. <em>pomonella</em>(^a) (n)</td>
<td>Petri Dish Pitfall Olfactometer</td>
</tr>
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<td>--------------------------</td>
<td>--------------------------------</td>
</tr>
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<td></td>
<td></td>
<td><strong>Pitfall 1</strong></td>
</tr>
<tr>
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<td></td>
<td>SB</td>
</tr>
<tr>
<td>35</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Pitfall 2</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Solvent</td>
</tr>
</tbody>
</table>

\(^a\) insects were allowed to cocoon on an open fluted cardboard (CB) strip 2.5 cm\(^2\);
\(^b\) CEE = cocoon extract equivalents;
\(^c\) CSLHE = cocoon-spinning larvae hour equivalents;
\(^d\) solvent consisted of redistilled pentane:ether (95:5) or pentane;
\(^e\) SB = decanal, nonanal, octanal, heptanal, (\(E\))-2-nonenal, (\(E\))-2-octenal, geranylacetone, sulcatone, 3-carene, myrcene, (+)-limonene;
\(^f\) 8-component blend consisted of SB minus (\(E\))-2-nonenal, myrcene, (+)-limonene;
\(^g\) male and female 5\(^{th}\) instar larvae
3.5. **Statistical Analyses**

Proportions of *M. ridibundus* adults and *C. pomonella* larvae responding to treatment and control stimuli in olfactometer bioassays were compared using the Chi-square goodness of fit test using Yates correction for continuity ($\alpha = 0.05$) to determine whether observed frequencies deviated significantly from expected frequencies under the null hypothesis that experimental insects did not have a preference for either treatment or control stimuli (Zar 1996).
4.0. RESULTS

4.1. Identification of Semiochemicals

GC-EAD analyses of Porapak Q extracts of airborne cocoon-derived semiochemicals revealed 10 volatiles that elicited responses from female *M. ridibundus* (Figure 2). EAD active compounds, plus an 11\textsuperscript{th} compound which was most abundant in the Porapak Q extract, were further subjected to GC-MS, and their identification confirmed by comparative GC, GC-MS, and GC-EAD analyses of *C. pomonella* produced and authentic standards. These 11 compounds were identified as: heptanal, 6-methyl-5-hepten-2-one [sulcatone], 7-methyl-3-methylene-1,6-octadiene [myrcene], octanal, 3,7,7-trimethylbicyclo[4.1.0]hept-3-ene [3-carene], 1-methyl-4-(1-methylene)-cyclohexene [(+)-limonene], (E)-2-octenal, nonanal, (E)-2-nonenal, decanal, and trans-6,10-dimethyl-5,9-undecadien-2-one [geranylacetone].

4.2. Bioassay Experiments with Natural and Synthetic Semiochemicals

Observations by Tom Unruh (personal communication) suggested the possibility that *M. ridibundus* may become entrained to cardboard odours upon emergence from codling moth cocoons in cardboard rolls. Unpublished experiments (Jumean) tested the attractiveness of cardboard versus no stimulus in Y-tube olfactometers revealing that naïve *M. ridibundus* females are not preferentially attracted to clean cardboard. This indicated that cardboard odours did not affect the parasitoids' decision and that cardboard could be used as a control stimulus.
Figure 2  Flame ionization detector (FID) and electroantennographic detector (EAD: female *Mastrus ridibundus* antenna) responses to aliquots of 10 CSLHE of Porapak Q extract of airborne volatiles from *Cydia pomonella* cocoons (1-3-d-old). Note: 1) 10 CSLHE (cocoon-spinning larvae hour equivalents = volatiles released from 10 cocoon-spinning *C. pomonella* larvae during 1 hr of cocoon-spinning activity); 2) antennal responses to 3-carene and myrcene (two additional candidate semiochemicals) not visible in the depicted trace; 3) although (+)-limonene was not EAD-active, it was the most abundant chemical in Porapak Q extracts and as such was included in behavioural experiments.
In separate experiments, cocoons (3-d-old) but not larvae or prepupae of *C. pomonella* attracted female *M. ridibundus* (Figure 3; experiments 1-3). Attractiveness of cocoons was not enhanced by the presence of prepupae (Figure 3; experiment 4), and depended upon the age of cocoons. Three-day-old cocoons containing late instar/prepupal *C. pomonella* were attractive (Figure 4; experiment 5) but 7- or 13-day-old cocoons containing *C. pomonella* pupae were not (Figure 4; experiments 6, 7). Similarly, 3-day-old cocoons were more attractive than 7- and 13-day-old cocoons (Figure 4, experiments 8, 9) in separate experiments although the former was not statistically significant.

Fresh (2 hr-old), unlike aged (8-d-old) MeOH extracts of 3-day-old cocoons attracted *M. ridibundus* (Figure 5; experiments 10, 11). Methanol washed cocoons were not attractive to *M. ridibundus* (Figure 5; experiment 12). Both 5- and 17-day-old Porapak Q extracts of airborne cocoon volatiles at aliquots of 1 CSLHE attracted *M. ridibundus*, whereas aliquots of 50 CSLHE did not (Figure 5; experiments 13-15).

The complete blend of 11 synthetic candidate semiochemicals (highlighted on figure 2) at 1 CSLHE strongly attracted *M. ridibundus* (Figure 6; experiment 16). The blend lacking either all aldehydes (experiment 17), unsaturated aldehydes (experiment 18), saturated aldehydes (experiment 19), ketones (experiment 20), or monoterpenes (experiment 21) was as unattractive as a pentane control. Deleting a single component from the 11-component blend in experiments 22-32 (Figure 7) determined that an 8-component blend (heptanal, octanal, nonanal, decanal, \((E)-2\)-octenal, sulcatone, geranylacetone, and 3-carene) is required to strongly attract *M. ridibundus*. Neither Porapak Q cocoon extracts nor the 8-component semiochemical blend differentially
Figure 3  Anemotactic response of female *Mastrus ridibundus* in Y-tube olfactometer experiments 1-4 to test stimuli consisting of either five *Cydia pomonella* cocoons (1-3-d-old), five larvae/prepupae excised from cocoons or both. Strips of corrugated cardboard (CB) served as pupation site. Number of insects responding to each stimulus given within bars; number of insects not responding in each experiment given in parentheses; asterisks indicate a significant response to a particular treatment; $\chi^2$ test with Yates correction for continuity; *P*<0.005.
EXP. 1

- Cocoon: 28% (4) *
- No stimulus: 8%

EXP. 2

- Cocoon + CB: 23% (11) *
- CB: 7%

EXP. 3

- Prepupa + CB: 20% (5)
- CB: 15%

EXP. 4

- Cocoon + prepupa + CB: 18% (8)
- Cocoon + CB: 14%
Figure 4  Anemotactic response of female *Mastrus ridibundus* in Y-tube olfactometer experiments 5-9 to test stimuli consisting of five *Cydia pomonella* cocoons each containing either a larval/prepupa or pupa, with or without corrugated cardboard strips (CB) that had served as pupation site. Number of insects responding to each stimulus given within bars; number of insects not responding in each experiment given in parentheses; asterisks indicate a significant response to a particular treatment; $\chi^2$ test with Yates correction for continuity; *P*<0.025.
EX'5  3-d-old cocoon
+ larva/prepupa + CB
   CB  10%  26% (4) *

EX'6  7-d-old cocoon
+ pupa + CB
   CB  16%  (8) 18%

EX'7  13-d-old cocoon
+ pupa + CB
   CB  14%  17% (9) 16%

EX'8  3-d-old cocoon
+ larva/prepupa + CB
7-d-old cocoon
+ pupa + CB
   CB  19%  12% (9)

EX'9  3-d-old cocoon
+ larva/prepupa + CB
13-d-old cocoon
+ pupa + CB
   CB  23%  9% (8) *
Figure 5  Anemotactic response of female *Mastrus ridibundus* in Y-tube olfactometer experiments 10-15 to MeOH cocoon extracts, MeOH extracted cocoons, or Porapak Q extracts of airborne cocoon-derived volatiles. Number of insects responding to each stimulus given within bars; number of insects not responding in each experiment given in parentheses; asterisks indicate a significant response to a particular treatment; $\chi^2$ test with Yates correction for continuity; *P<0.05; **P<0.01; ***P<0.005. Note: 1) cocoons were 1-3 days old at the time of extraction or aeration but MeOH cocoon extracts or Porapak Q extracts were tested before and after aging to determine stability of semiochemicals; 2) the amount of stimulus tested was equivalent to 20 CEE (cocoon-extract equivalents = MeOH extract of 20 *Cydia pomonella* cocoons), and 1 or 50 CSLHE (cocoon-spinning larvae hour equivalents = semiochemicals produced by 1 or 50 cocoon-spinning *C. pomonella* larva(e) during 1 hr of cocoon-spinning activity); 3) the same amount of solvent was applied to treatment and control stimuli.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Description</th>
<th>Test stimuli</th>
<th>Number of insects responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXP. 10</td>
<td>MeOH cocoon extract (1-hr-old)</td>
<td>0 2 4 6 8 10 12 14 16 18 20</td>
<td>14 (2) *</td>
</tr>
<tr>
<td>EXP. 11</td>
<td>MeOH cocoon extract (8-d-old)</td>
<td>0 2 4 6 8 10 12 14 16 18 20</td>
<td>10 (2)</td>
</tr>
<tr>
<td>EXP. 12</td>
<td>MeOH washed cocoons</td>
<td>0 2 4 6 8 10 12 14 16 18 20</td>
<td>11 (3)</td>
</tr>
<tr>
<td>EXP. 13</td>
<td>Porapak Q extract (1 CSLHE, 5-d-old)</td>
<td>0 2 4 6 8 10 12 14 16 18 20</td>
<td>17 (1) ***</td>
</tr>
<tr>
<td>EXP. 14</td>
<td>Porapak Q extract (1 CSLHE, 17-d-old)</td>
<td>0 2 4 6 8 10 12 14 16 18 20</td>
<td>16 (1) **</td>
</tr>
<tr>
<td>EXP. 15</td>
<td>Porapak Q extract (50 CSLHE)</td>
<td>0 2 4 6 8 10 12 14 16 18 20</td>
<td>8 (7)</td>
</tr>
</tbody>
</table>
Figure 6  Anemotactic response of female *Mastrus ridibundus* in Y-tube olfactometer experiments 16-21 to a 11-component blend of synthetic candidate semiochemicals (SB), or SB lacking groups of organic chemicals. Number of insects responding to each stimulus given within bars; number of insects not responding in each experiment given in parentheses; asterisks indicate a significant response to a particular treatment; $\chi^2$ test with Yates correction for continuity; *$P<0.01$. Note: 1) SB consisted of 3 monoterpenes [(+)-limonene, 3-carene, myrcene], 4 saturated aldehydes [heptanal, octanal, nonanal, decanal], 2 unsaturated aldehydes [(E)-2-octenal, (E)-2-nonenal], and 2 ketones [sulcatone, geranylacetone]; 2) aliquots of 1 CSLHE (see caption of figure 2) were tested; 3) the same amount (5 μl) of pentane was applied to treatment and control stimuli.
<table>
<thead>
<tr>
<th>Exp.</th>
<th>Test Stimuli</th>
<th>Number of Insects Responding</th>
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<tbody>
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<td>16</td>
<td>SB</td>
<td>15 (2) *</td>
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<tr>
<td></td>
<td>Solvent control</td>
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</tr>
<tr>
<td>17</td>
<td>SB minus all aldehydes</td>
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<td></td>
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</tr>
<tr>
<td>18</td>
<td>SB minus unsaturated aldehydes</td>
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<td>Solvent control</td>
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<td>19</td>
<td>SB minus saturated aldehydes</td>
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<tr>
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<td>SB minus ketones</td>
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<td>21</td>
<td>SB minus monoterpenes</td>
<td>12 (3)</td>
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<tr>
<td></td>
<td>Solvent control</td>
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</tr>
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</table>
Figure 7  Anemotactic response of female *Mastrus ridibundus* in Y-tube olfactometer experiments 22-32 to a 11-component blend of synthetic candidate semiochemicals (SB; see also caption of figure 6) or SB lacking individual components. Number of insects responding to each stimulus given within bars; number of insects not responding in each experiment given in parentheses; asterisks indicate a significant response to a particular treatment; \( \chi^2 \) test with Yates correction for continuity; *P<0.025; **P<0.01. Note: 1) Aliquots of 5 CSLHE (see caption of figure 2) were tested; 2) the same amount (5 \( \mu \)l) of pentane was applied to treatment and control stimuli.
| EXP. 22 | SB minus decanal | Solvent control | 8 | 3 | (3) |
| EXP. 23 | SB minus nonanal | Solvent control | 9 | 3 | (3) |
| EXP. 24 | SB minus octanal | Solvent control | 5 | 3 | (1) |
| EXP. 25 | SB minus heptanal | Solvent control | 10 | 3 | (2) |
| EXP. 26 | SB minus (E)-2-nonenal | Solvent control | 4 | 3 | (1) * |
| EXP. 27 | SB minus (E)-2-octenal | Solvent control | 8 | 3 | (2) |
| EXP. 28 | SB minus geranylacetone | Solvent control | 9 | 3 | (2) |
| EXP. 29 | SB minus sulcatone | Solvent control | 11 | 3 | (0) |
| EXP. 30 | SB minus 3-carene | Solvent control | 12 | 3 | (2) |
| EXP. 31 | SB minus myrcene | Solvent control | 3 | 3 | (3) * |
| EXP. 32 | SB minus (+)-limonene | Solvent control | 16 | 3 | (1) ** |

<table>
<thead>
<tr>
<th>Test stimuli</th>
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</tr>
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<tbody>
<tr>
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<td>2</td>
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<tr>
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attracted *M. ridibundus* over the complete 11-component semiochemical blend (Figure 8; experiments 33, 34).

The 11-component synthetic semiochemical blend tested at 200 CSLHE attracted/arrested pupation site seeking fifth instar *C. pomonella* (Figure 9; experiment 35).
Figure 8  Anemotactic response of female *Mastrus ridibundus* in Y-tube olfactometer experiments 33-34 to test stimuli consisting of either the 11-component blend of synthetic candidate semiochemicals (SB; see also caption of figure 6), Porapak Q extracts of airborne cocoon-derived volatiles, or the 8-component blend of semiochemicals. Number of insects responding to each stimulus given within bars; number of insects not responding in each experiment given in parentheses. Note: 1) the 8-component blend consisted of one monoterpenel [3-carene], four saturated aldehydes [heptanal, octanal, nonanal, decanal], one unsaturated aldehyde [(E)-2-octenal], and two ketones [sulcatone and geranylacetone]; 2) aliquots of 5 CSLHE (see caption of figure 2) were tested.
EXPERIMENT 33

- Porapak Q extract: 6 insects responding
- 8-component blend: 10 insects responding

EXPERIMENT 34

- 11-component blend: 8 insects responding
- 8-component blend: 11 insects responding

Test stimuli vs. Number of insects responding
Figure 9  Response of 5th instar *Cydia pomonella* larvae in Petri dish pitfall olfactometer (Duthie *et al*. 2003) experiment 35 to either a 11-component blend of semiochemicals (SB; see caption of figure 6) or a solvent (pentane) control stimulus. Number of larvae responding to each stimulus given within bars; number of larvae not responding in experiment 35 given in parentheses; asterisks indicate a significant response to a particular treatment; $\chi^2$ test with Yates correction for continuity; *P*<0.001. Note: the same amount (25 µl) of pentane was applied to treatment and control stimuli.
EXP. 35

SB

Solvent control

Number of insects responding

Test stimuli

0 5 10 15 20 25 30 35

0 5 10 15 20 25 30 35

(5)*
5.0. DISCUSSION

My data support the hypothesis that semiochemicals from cocoon-spinning *C. pomonella* larvae attract host-foraging *M. ridibundus*. This conclusion is based on the findings that *M. ridibundus* were attracted to a) cocoons containing *C. pomonella* late instar larvae or prepupae (Figure 3; experiment 1); b) fresh MeOH cocoon extract (Figure 5; experiment 10); and c) Porapak Q extract of cocoon-derived airborne semiochemicals (Figure 5; experiments 13, 14).

Moreover, attraction of *M. ridibundus* to *C. pomonella* cocoons (Figure 3; experiments 1, 3), equal attraction to cocoons with or without insects inside, and no attraction to excised *C. pomonella* prepupae (Figure 3, experiments 2, 4), all strongly suggest that the semiochemicals are associated with the cocoon.

Attractiveness of cocoons containing *C. pomonella* late instar larvae or prepupae, and lack of attractiveness of cocoons containing pupae (Figure 4; experiments 5-7) further indicate that the prepupal parasitoid *M. ridibundus* exploit host semiochemicals not only to locate hosts, but also to locate hosts in the stage of development suitable for parasitism. While cocoon-derived semiochemicals have been implied as contact host recognition cues for parasitoids (Weseloh 1977, 1981; Bekkaoui and Thibout 1993; Benedet et al. 1999), my data show that cocoon-derived airborne semiochemicals serve as long-range attractants to *M. ridibundus*.

Some constituents of this semiochemical blend have also been reported to elicit antennal or behavioural responses by other parasitic wasps. Heptanal elicits antennal
activity by the braconid parasitoid *Microplitis croceipes* (Cresson) (Li et al. 1992), and nonanal and geranylacetone attract the clothes moth parasitoid *Apanteles carpatus* to host habitat (Takács et al. 1997). Further, octanal, nonanal, and decanal derived from stink bug abdominal glands may serve as semiochemicals for parasitic Tachinids (Diptera) and Scelionids (Hymenoptera) (Aldrich 1995). Finally, heptanal, octanal, nonanal, and decanal emanating from scales of *C. pomonella* serve as semiochemicals to *A. quadridentata*, a braconid egg parasitoid of *C. pomonella* (DeLury et al. 1999).

Response of *C. pomonella* larvae seeking pupation sites to the 11-component semiochemical blend (Figure 9; experiment 35) reveals that one or more of these semiochemicals serve as an aggregation pheromone (Duthie et al. 2003) for *C. pomonella* larvae. Obviously, female *M. ridibundus* eavesdrop on the pheromonal communication of cocoon-spinning *C. pomonella* larvae to locate host prepupae. The pheromone is effective, and most attractive to *M. ridibundus*, during the larval cocoon-spinning stage (Duthie et al. 2003; this study) but remains detectable in the prepupal stage (Figure 4; experiments 8, 9). Degradation or dissipation of the pheromone within 3 days after cocoon-spinning activity has ceased appears adaptive to both *C. pomonella* larvae and female *M. ridibundus*. If pheromone-based aggregation of *C. pomonella* larvae is a strategy to procure and synchronize development of future mates (Duthie et al. 2003), fading attractiveness of aging cocoons prevents continued attraction of larvae and asynchronous eclosion of potential mates. This characteristic of the semiochemical signal also makes it a reliable indicator of host presence. *Mastrus ridibundus* as a specialist of cocoon-spinning *C. pomonella* larvae (Isankalova 1972; Abdullaev 1974;
Kuhlman and Mills 1999) seem to use these temporal dynamics of the semiochemicals to determine the proper late instar/prepupal stage of the host.

Semiochemicals also facilitate foraging in patches with high probability of host encounter (Chiri and Legner 1982, 1986). If the semiochemical concentration is proportional to host density, *M. ridibundus* may estimate host density, and thus patch profitability, even before encountering a host insect (van Alphen and Vet 1986; Geervliet et al. 1998; Wertheim et al. 2003). Moreover, if foraging females can detect variation in host density on trees, hosts on trees supporting high densities of *C. pomonella* larvae are at a greater risk of being discovered and parasitized by *M. ridibundus* (Bezemer and Mills 2001).

For *C. pomonella* larvae, pheromone-based aggregation may represent a trade-off between procuring future mates (Duthie et al. 2003), and the risk of attracting parasitoids. For example, Bezemer and Mills (2001) provided evidence that female *M. ridibundus* search and oviposit more frequently on trees with high rather than low host densities. Similarly, Roitberg and Lalonde (1991) demonstrated that female rosehip flies, *Rhagoletis basiola*, oviposit on and mark the rosehips with semiochemicals which in turn facilitates egg location and increases the risk of egg parasitism by the pteromalid wasp *Halticoptera rosae*. However, if *M. ridibundus* females are egg-limited (Bezemer and Mills 2001), then the probability of parasitism may be lower for *C. pomonella* larvae cocooning in aggregates than for those cocooning singly.

Information conveyance plays an important role in host-parasitoid interactions. Female *M. ridibundus* seem to have solved the reliability-detectability challenge of host signals by eavesdropping on the pheromonal communication of host larvae/prepupae.
Host pheromones are most suitable signals for foraging parasitoids because pheromones tend to be detectable and reliable indicators of host presence (Wiskerke et al. 1993; Wertheim et al. 2003). The reliability of the cocoon-derived signal seems to be based on its complexity. Eight compounds from four classes of organic chemicals were required to elicit a strong behavioural response of *M. ridibundus* (Figure 6, experiments 17-21). It will now be intriguing to investigate whether the aggregation pheromone of *C. pomonella* larvae comprises an equally complex signal.
6.0. REFERENCES

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