COMMUNICATION OF CODLING MOTH LARVAE: IDENTIFICATION AND FUNCTIONAL ROLE OF THEIR AGGREGATION PHEROMONE

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ABSTRACT

Pupation site-seeking larvae of the codling moth, *Cydia pomonella* (L.), (Lepidoptera: Tortricidae), aggregate in response to pheromone emanating from freshly spun cocoons of conspecific larvae. My main objectives were to identify the pheromone and to study its functional roles.

In unmanaged apple orchards I surveyed tree trunks for cocooning larvae and found aggregates of larvae significantly more often than solitary larvae.

In 31 two-choice olfactometer experiments, a pheromone blend of (E)-2-octenal, (E)-2-nonenal, sulcatone, and geranylacetone, in combination with either 3-carene and/or three saturated aldehydes (octanal, nonanal, decanal), elicited behavioural responses from fifth-instar larvae.

Both male and female larvae produced, and responded to, aggregation pheromone.

Larvae were attracted to, rather than merely arrested by, larval aggregation pheromone. They moved faster and farther upwind toward cocooning conspecifics compared to blank controls, and selected more often as first- and final choices of pupation sites those with cocooning conspecifics than those without. Finally, in Y-tube olfactometers, they anemotactically responded to, and preferred side arms with, cocooning conspecifics to those without.
Aggregation behaviour by larvae does not appear to increase the rate of parasitism. In a wind tunnel, 10 larvae in aggregations were more readily located by female parasitoids *Mastrus ridibundus* than 10 larvae well separated from each other. Larval cocooning in aggregation or isolation had no effect on the mean rate of parasitism and the mean number of eggs deposited per parasitized host. The increased risk of aggregated larvae to be detected by egg-limited *M. ridibundus* is likely offset by diluted parasitism risk.

A genetic algorithm model predicted that larval aggregation behaviour is selected for when the probability of mate encounter away from emergence sites is low, the time to locate mates is short, and *M. ridibundus* are at high population levels.

In field experiments, corrugated cardboard bands treated with synthetic pheromone were more effective in capturing *C. pomonella* larvae than untreated bands, providing proof of concept that synthetic pheromone can be used to enhance captures of fifth-instar larvae in trapping devices.

Mature larvae of Oriental fruit moth, *Grapholita molesta*, and Indianmeal moth, *Plodia interpunctella*, were not attracted to, or arrested by, cocoon-spinning conspecifics.
Keywords: Cydia pomonella, larva, aggregation pheromone, behaviour, benefits and costs, pest management
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1: INTRODUCTION
1.1 General introduction

Animal aggregations are a common phenomenon and have significant implications at the individual level and at population and community levels. Aggregation behaviour can evolve if the fitness accrued by an individual through grouping with others is greater than the fitness accrued by remaining solitary (Parrish and Edelstein-Keshet 1999). More specifically, an individual will group (aggregate) with others until a threshold number of conspecifics within an aggregation is reached at which time the fitness of all individuals is optimized. With each new individual that joins an aggregation past this threshold, an individuals' fitness will decrease incrementally until the fitness of each individual equals that of one that remains solitary (Giraldeau and Caraco 2000). Animal aggregations are known from a wide range of taxa including mammals, birds, fish, and invertebrates. My thesis focuses on aggregations in insects, building on a former conclusion that the mechanisms and functionality of aggregations are analogous among taxa (Wertheim et al. 2005).

As with any decision an animal might make, there are costs and benefits associated with the decision to join a group of con- or heterospecifics (Krause and Ruxton 2002; Wertheim et al. 2005). Benefits accrued by individuals within an aggregation have been well studied and can include mate finding (Wertheim et al. 2002; Verhoef and Nagelkerke 1977), efficient extraction of resource nutrients (Phillips and Strand 1994; Krause and Ruxton 2002), overcoming plant
defenses (Raffa 2001; Wertheim et al. 2002), protection from natural enemies (Lockwood and Story 1986; Turner and Pitcher 1986; Wrona and Dixon 1991), or protection from adverse environmental conditions (Lockwood and Story 1986). Costs associated with the grouping of individuals may include increased competition for resources (Raffa 2001; Bjørnstad et al. 1998), increased conspicuousness to natural enemies (Wertheim et al. 2003), or deterioration of environmental condition (Geervliet et al. 1998).

Most research on insect aggregation behaviour has focused either on aggregations of mixed-stage hemimetabolous insects (Lockwood and Story 1986; Nishida et al. 1993; Siljander et al. 2007) and on adult or adult-mediated aggregations in holometabolous insects (Wertheim et al. 2002). The few studies of larval-mediated aggregations include the great spruce bark beetle, *Dendroctonus micans* (Deneubourg et al. 1990; Storer et al. 1997), the blister beetle, *Meloe franciscanus* (Saul-Gershenz and Millar 2006), and the Indianmeal moth, *Plodia interpunctella* (Anderson and Löfqvist 1996). This paucity of studies suggests that larval-mediated aggregations are rare.

Previously, Duthie et al. (2003) revealed that larvae of the codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), aggregate in response to signals produced by cocoon-spinning conspecific larvae. However, the mechanism of these larval aggregations and their implications for *C. pomonella* biology remained unknown. The ultimate aim of my thesis is to contribute to our understanding of animal aggregations by studying the behavioural and chemical ecology of larval aggregations in *C. pomonella*. Specifically, I describe the
chemical identity of the larval aggregation pheromone and the mechanisms by which larval aggregation occurs. I offer an ecological cost-benefit perspective to provide a set of functional explanations for larval aggregation. Finally, I evaluate the use of synthetic larval aggregation pheromone as a tool for the management of *C. pomonella* larvae in an orchard environment. I have obtained results through a combination of laboratory analyses and bioassays, field experiments, and theoretical modelling.

### 1.2 Overview of thesis chapters

My thesis is organized into nine chapters. Following a brief introductory chapter, there are eight research chapters and a final concluding chapter summarizing the major findings. The dissertation is organized as an article-style thesis. Research chapters closely resemble manuscripts that have already been published (Chapters 2-6, 8-9) or will soon be submitted for review (Chapter 7). Each research chapter (manuscript) is presented in the style and format prescribed by the journal that has published the manuscript and typically comprises an abstract, introduction, materials and methods, results, discussion and a reference list. Figures and tables are presented at the end of each chapter. In the following section, I briefly outline the content of each research chapter.

In Chapter 2, I studied the phenomenon of *C. pomonella* larval aggregation *in situ*. Larval aggregations have been studied in the laboratory (Duthie et al. 2003) but their occurrence and frequency distribution in the field had not previously been investigated. Thus, I conducted surveys in abandoned
apple orchards, carefully checking for the presence and group size of larvae on specific sections of trees. I have found that aggregates of larvae occurred significantly more often than solitary larvae, and that the number of cocooning larvae in aggregates (= group size) was inversely correlated with the frequency occurrence of that group size.

In Chapter 3, I test the hypothesis that both male and female *C. pomonella* larvae produce and respond to the aggregation pheromone. In dual-choice olfactometer experiments using coccon-spinning male or female larvae as test stimuli, and pupation-seeking male or female larvae as bioassay insects, I have demonstrated that both male and female larvae produce and respond to aggregation pheromone.

In Chapter 4, I build on results of previous chapters and report the components of the aggregation pheromone. Through a series of dual-choice olfactometer experiments, I have determined that eight components, out of 13 candidate compounds, comprise the complex pheromone blend and that synthetic aggregation pheromone is as attractive as larvae-produced aggregation pheromone.

In Chapter 5, I investigate the mechanism of larval aggregation. In a series of behavioural laboratory bioassays, I tested whether the pheromone induces attraction or arrestment by fifth-instar pupation-site-seeking larvae. I have shown that the pheromone acts as a long-range attractant, eliciting a direct-guiding response by pupationsite-seeking larvae from a distance of greater than 30 cm.
In Chapter 6, I investigate whether larval aggregation behaviour has fitness costs inflicted by the parasitoid *Mastrus ridibundus* that exploits the larval aggregation pheromone as a host location kairomone (Jumean et al. 2005). I tested whether aggregations of various sizes and structures incur costs to the aggregating individuals. I have shown that larvae in aggregations are more conspicuous to foraging *M. ridibundus* but are less likely to be parasitized.

In Chapter 7, I explore ecological parameters whereby selection might favour the evolution of larval aggregation behaviour. Applying genetic algorithms, I have shown that larval aggregation behaviour can be selected for when parasitoid pressure is high, the period of mating opportunities is brief, and the probability is low that mates find each other in locations other than those of larval aggregations.

In Chapter 8, I test the concept of using synthetic aggregation pheromone for mass trapping *C. pomonella* larvae in commercial apple orchards. I affixed cardboard bands with or without pheromone impregnation to apple tree trunks and compared the number of larvae that cocooned in treatment and control bands. I have shown that bands treated with synthetic pheromone attracted significantly more larvae for cocooning than control bands, suggesting that pheromone-based mass trapping of larvae has potential to become part of integrated programs for control of *C. pomonella* populations in commercial apple orchards.

In Chapter 9, I explore whether larvae of the closely related Oriental fruit moth, *Grapholita molesta*, and of the Indianmeal moth, *Plodia interpunctella*, both
of which share similar life-history strategies with *C. pomonella*, also produce an aggregation pheromone before pupating. In various behavioural bioassays, I have shown that neither Oriental fruit moth nor Indianmeal moth larvae produce aggregation pheromones, indicating that the aggregation of *C. pomonella* larvae appears to be a rare phenomenon.

In my concluding chapter, I summarize my key findings and propose future research directions.

1.3 *Cydia pomonella* life history

The peak flight activity of *C. pomonella* occurs just prior to and after sunset and, under favourable conditions, may last for up to 2 h (Dolstad 1985; Howell et al. 1990). Optimal temperatures for flight range from 15-27°C, above and below which flight activity slows down or ceases (Dolstad 1985). At dusk, females “call” potential mates by releasing a long-range sex pheromone blend comprising the primary component (E,E)-8,10-dodecadienol (Roelofs et al. 1971) and the 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 between 30-50 eggs (Geier 1963; Pedigo 1999; Unruh and Lacey 2000) singly directly on fruit of the host plants (e.g. apple, pear, walnut, quince) or near the fruit on plant foliage (Summerland and Steiner 1943;
Thiery et al. 1995; Landolt et al. 1999). The freshly deposited egg is white and convex, and appressed close to the substrate (Dolstad 1985). As the embryo develops, the egg darkens and a red ring surrounds the developing insect. Finally, 1-2 d before hatching, the head capsule of the larva becomes visible through the egg as a black spot (Dolstad 1985). Neonate larvae hatch 5-15 d following oviposition and commence foraging for fruit within a few hours (Pedigo 1999).

Larvae complete five instars before pupating (Figure 1.1). The white first instar larva is approximately 1.5 mm in length with a black head capsule, whereas the mature fifth-instar larva is approximately 20 mm in length with a brown head capsule and cervical shield, and a pinkish body (Dolstad 1985). Mature male larvae are slightly smaller than mature female larvae and can be distinguished from female larvae by the presence of dark spots (the future testes) on their mid-dorsal surface (Jumean et al. 2009).

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 typically burrow in through the calyx leaving a “sting mark”. They continue to feed on the flesh of the fruit until they reach the core within which they feed on 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 instars. Mature fifth instars burrow out of the apple fruit, sometimes creating a second sting mark, and seek cryptic and protective microhabitats including cracks or crevices in the trees’ bark and
ground litter where they spin a cocoon and pupate in aggregations (Geier 1963; Duthie et al. 2003, Jumean et al. 2004, 2009). The time from larval hatching to pupation takes 14-35 d (Geier 1963). Pupae are dark brown in colour and the pupal stage lasts 8-30 d (DeLury 1998).

*Cydia pomonella* is protandrous. In the laboratory, adult males eclose commonly a few hours (ZJ, unpubl. data) but up to 2 d (Duthie et al. 2003) before females. Adult moths are 6-7 mm in length 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 (Dolstad 1985; Pedigo 1999). At rest, the wings are held roof-like over the body (Dolstad 1985). Adult moths live for 14-21 d in the field (Pedigo 1999).

### 1.4 Literature cited


DeLury, N.C. 1998. Pheromonal and kairomonal attraction of *Ascogaster quadridentata* Wesmael (Hymenoptera: Braconidae), a parasitoid of
Cydia pomonella L. (Lepidoptera: Tortricidae). M.Sc. Thesis. Simon Fraser University, Burnaby, Canada.


Unruh, T.R. and Lacey, L.A. 2001. Control of codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae), with *Steinernema carpocapsae*: Effects of
supplemental wetting and pupation site on infection rate. *Biological Control*. 20: 48-56.


Figure 1.1  Life cycle of the codling moth, *Cydia pomonella*. 
Adult:
Lives 14 to 21 days

Pupa (inside cocoon):
14 to 30 days

Egg:
Hatches after 5 to 15 days

Larva (5 instars):
Feeds for 21 to 35 days.
2: FREQUENCY DISTRIBUTION OF LARVAL CODLING MOTH, *Cydia pomonella* (L.), AGGREGATIONS ON TREES IN UNMANAGED APPLE ORCHARDS OF THE PACIFIC NORTHWEST¹

2.1 Abstract

The codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), is a key pest of pome fruits in North America. Upon location of a pupation site, larvae spin a cocoon from which aggregation pheromone disseminates that attracts conspecific larvae. In two unmanaged apple orchards in Wenatchee and Yakima, Washington State, USA, we systematically surveyed cracks and crevices by peeling bark flakes off of tree trunks in search for cocooning *C. pomonella* larvae. Aggregates of larvae were found significantly more often than solitary larvae. The number of cocooning larvae in aggregates (= group size) was inversely correlated with the frequency occurrence of that group size. Group size ranged between 2-20 cocoons. Height above ground had no effect on location of aggregates. In Orchard 1, the cardinal direction of the tree trunk had no effect on location of aggregations, but in Orchard 2 aggregations were located significantly most often on the south side of trunks. The mean ratio of males and females in aggregations was 1.08:1 and 1.04:1 in Orchards 1 and 2, respectively. Moreover, the number of males in aggregates did not significantly differ from that of females. Our data support the conclusion that larvae seek pupation sites not by chance but in large part in response to pheromone signal and microhabitat cues. The probability of aggregates forming is likely proportional to the population density of *C. pomonella*. 
2.2 Introduction

In many insect taxa, individuals form groups or aggregations in response to signals or cues from conspecifics or resources (Prokopy and Roitberg 2001; Krause and Ruxton 2002) including potential mates, food, oviposition sites or shelters. Individuals in aggregations benefit through reduced mortality by natural enemies, increased probability of finding food or mates, or improved ability to overcome plant defenses (Prokopy and Roitberg 2001).

In non-social holometabolous insects, pheromone-based aggregations are common among adults but rare among larvae, or larvae and adults. Only a few such communication systems have been reported. The larval pheromone of the Indianmeal moth, *Plodia interpunctella*, induces oviposition by adult females which results in larger larval populations that more efficiently utilize resource nutrients, reduce microbial growth on food, and generate a larger matting of silk as a means of protection from natural enemies (Phillips and Strand 1994; Prokopy and Roitberg 2001). At high larval densities and thus high concentrations of pheromone, however, the pheromone repels larvae, thereby regulating population density at a resource (Mossadegh 1980, Prokopy and Roitberg 2001). Larvae of the bark beetle, *Dendroctonus micans*, produce a pheromone which facilitates aggregative and efficient feeding, with aggregated individuals growing faster and larger than solitary ones (Storer et al. 1997).

Larvae of the codling moth, *Cydia pomonella* (L.), produce a pheromone while spinning cocoons which attracts conspecific larvae to the site of pheromone
release (Duthie et al. 2003; Jumean et al. 2004, 2005a, b, 2008). *Cydia pomonella* is the most important pest of apple fruits, other pome fruits and nuts worldwide (Clausen 1978). In the western USA and British Columbia, Canada, it is the key pest of apple (Calkins and Faust 2003; Judd et al. 1997). Damage is caused when first-instar larvae bore into apples, causing diagnostic “sting marks”. Larvae develop through five instars. Fully mature fifth-instar larvae exit the fruit either through the entrance tunnel or a new tunnel and traverse a host tree trunk in search of a suitable site in which to spin a cocoon and pupate. The aggregation pheromone disseminates from freshly spun cocoons of male and female larvae attracting other male and female larvae (Duthie et al. 2003; Jumean et al. 2004, 2005a,b, 2008). Compared to solitary larvae, aggregated larvae are better protected from parasitism by the parasitic wasp *Mastrus ridibundus* through dilution effects and the structural refugia that they create (Jumean et al. 2009b).

Larvae respond to aggregation pheromone in the laboratory (Duthie et al. 2003, Jumean et al. 2005a) and to pheromone-baited cardboard bands in managed orchards (Jumean et al. 2007). However, the occurrence and characteristics of aggregations of *C. pomonella* larvae in orchard settings have not yet been intensely studied. Closing this knowledge gap would shed light on the significance of larval communication and aggregation, and help design management tactics that target larvae in addition to adults. Here, we report group size, sex ratio, location and frequency distribution of larval/pupal aggregates on trees in two unmanaged apple orchards.
2.3 Materials and methods

2.3.1 Survey orchards

Surveys were conducted in two, 2.5-ha abandoned apple orchards (Orchard 1: N 47˚26.268 W 120˚21.389; Orchard 2: N 46˚58.279 W 120˚35.431) in Wenatchee and Yakima, Washington State, USA, respectively. Each orchard had moderate to high population densities of *C. pomonella*. Orchards were 25-40 years old and planted at a density of ~200 trees/ha, with a tree × row spacing of 3 × 5 m (Orchard 1) and 4 × 5 m (Orchard 2). The apple variety in Orchard 1 was “Golden Delicious”, and the varieties in Orchard 2 were “Golden Delicious” and “Delicious”. Trees in Orchard 1 and Orchard 2 had a mean circumference at 50 cm above ground of ~43 and ~83 cm, respectively. The gross features of the bark (presence of numerous cracks, crevices, and fissures) and thus the quality of the microhabitat for *C. pomonella* larvae were comparable within and between orchards. Surveys were conducted between 20-27 October, 2006 when aggregations of the diapausing, overwintering generation were expected to be most prevalent.

2.3.2 Sampling protocol

Selecting every other tree, we surveyed a total of 35 trees in Orchard 1 and 25 trees in Orchard 2 for the presence of *C. pomonella* larvae. We surveyed the lowest 1-m trunk section because our exploratory surveys of entire trees had yielded few larvae above this height. Three trees on either end of each row within
blocks were excluded to minimize edge effects as adjacent blocks had received different treatments, which might have affected the presence of *C. pomonella* at the edges of our survey blocks. Aggregation was defined as ≥2 occupied cocoons with at least one in physical contact with another. In sequence, we (1) searched the surface of a trunk and fissures on the surface for exposed and visible cocoons; (2) gently tore such cocoons apart with fine forceps to determine occupancy; (3) inspected the soil-tree interface for cocoons; (4) peeled off bark flakes with sturdy knives to expose larvae beneath; and (5) lifted or removed tightly attached sections of bark to expose cocooned larvae in such spaces. For each larva, we recorded: (1) the gender (gonads are visible on the mid-dorsal surface of male larvae), (2) the gender and number of additional larvae; (3) cardinal direction and (4) height above ground (in 10-cm partitions). Potential pupation sites as perceived by the authors that housed zero larvae were not recorded. All larvae were collected to avoid repeated recordings.

### 2.3.3 Data analyses

For each orchard, the number of larvae cocooning singly or in aggregates was analyzed with the \( \chi^2 \) goodness-of-fit test with Yates correction for continuity. Differences in height or cardinal direction of pupation sites were analyzed with a \( \chi^2 \) goodness-of-fit test for multiple categories followed by subdivision of categories (Zar 1999). The mean sex ratio (male:female) of aggregations was determined by dividing the number of males with the total number of individuals in each aggregation, taking the mean of all aggregations, and converting it to a
ratio out of 1. The difference in numbers of females and males in aggregations was analyzed with a Wilcoxon signed rank paired-sample test (SAS version 9.1). The experimental error rate in all experiments was set at \( \alpha = 0.05 \).

### 2.4 Results

In both orchards, larvae were found to be cocooning singly or in aggregates of varying sizes (Figure 2.1). The number of cocoons in aggregates (= group size) was inversely correlated with the frequency occurrence of that group size (Figure 2.1). Overall, aggregated specimens were found more often than solitary specimens (Orchard 1: \( \chi^2_{0.05,1} = 18.14, P < 0.001, n = 127 \); Orchard 2: \( \chi^2_{0.05,1} = 31.20, P < 0.01, n = 195 \); Figure 2.2) but the number of solitary larvae encountered was greater than any other group size. Group size (excluding solitary larvae) ranged between 2-20 cocoons (mean = 3.33) in Orchard 1 and between 2-16 (mean = 3.48) in Orchard 2 (Table 2.1). In Orchard 1, the location of aggregations was independent of the cardinal sector of the tree trunk \( \chi^2_{0.05,3} = 5.60, P > 0.10, n = 76 \), but in Orchard 2 aggregations were located significantly most often on the south side of trunks \( \chi^2_{0.05,1} = 3.94, P < 0.05, n = 108 \); Table 2.1). Location of aggregations was independent of height above ground in Orchard 1 \( \chi^2_{0.05,7} = 13.03, P > 0.05, n = 73 \) and Orchard 2 \( \chi^2_{0.05,7} = 14.07, P > 0.05, n = 108 \). The mean ratio of males and females in aggregations was 1.08:1 and 1.04:1 in Orchards 1 and 2, respectively (Table 2.1). Moreover, the number of males in aggregates did not significantly differ from that of females (Orchard 1: \( P = 0.87, n = 34 \); Orchard 2: \( P = 0.47, n = 47 \)).
2.5 Discussion

Our data reveal that larvae of *C. pomonella* cocoon singly and in aggregates in the field (Figure 2.1). However, the number of individuals cocooning with at least one other individual is greater than the number of individuals cocooning by themselves. Considering that most tree trunks contained numerous unoccupied cracks, crevices, and bark scales that appeared to be suitable larval pupation sites (i.e. pupation sites are not limiting), these results suggest that aggregation may be facilitated by aggregation pheromone. These results contribute to our knowledge of cocooning behaviour and dynamics of *C. pomonella* in the field. Moreover, these results seem to support the concept that larvae locate pupation sites in response to both a pheromonal signal and microhabitat cues.

The search for pupation sites reportedly occurs in two distinct phases (Geier, 1963). In the displacement phase, the very mobile larvae readily traverse tree trunks. This displacement phase is punctuated by an area-searching phase, often when larvae encounter a trunk section with suitable pupation sites (Geier, 1963). In the area-searching phase, larvae typically stop and display characteristic head thrusts (Geier, 1963, Z.J. pers. obs.), possibly probing for the presence of aggregation pheromone at prospective pupation sites. With the active range of the pheromone exceeding 20 cm (Jumean et al., 2008), larvae are likely to detect airborne pheromone from cocoon-spinning conspecifics nearby. A combination of chemo-orthokinetic and tactic responses (Jumean et
al., 2008) might then guide foraging larvae to the site(s) of pheromone release, resulting in aggregates of cocooning larvae.

This concept is equally applicable to female and male larvae, because they occurred in aggregation at a sex ratio of 1:1 (Table 2.1), and both female and male larvae produce and respond to aggregation pheromone (Jumean et al. 2004). The concept that aggregations may form in response to both pheromone and physical characteristics of potential pupation sites is supported by observations that at least 50% of sites, most of which were best suited to host a single larva, were unoccupied (Z.J. pers. obs.), suggesting that pupation sites are not a limiting resource. Interestingly, larvae were either absent from, or present in numbers of ≥2 on each trunk surveyed.

The mean number of larvae in aggregates (3.33 and 3.48 in Orchard 1 and 2, respectively) is a conservative estimate based on our strict protocol that larvae must be in physical contact with one another to be considered aggregated. There were numerous observations of cocoons or groups of cocoons that were separated by as little as 1 cm. When considering the proposed benefits (e.g., efficient location of pupation sites, expedient adult mating, reduced risk of parasitism) and costs (e.g., increased rate of location by parasitoids) (Jumean et al., 2009) of pheromone-based larval aggregation, insects occupying cocoons in close proximity to other cocoons would still experience the same selective pressures as insects occupying cocoons in physical contact with one another. The insects themselves define what constitutes an aggregation’s boundaries
therefore a close look at the insects’ ecology and behaviour will help us to better define larval aggregations (Hassell and Southwood 1978).

Neither height above ground nor cardinal direction had profound effects on selections of pupation sites (Table 2.1), suggesting that other physical parameters of prospective pupation sites provide stronger foraging cues. Thigmotactic stimuli associated with cracks and crevices may constitute such cues (Geier, 1963) and may be critically important in selecting a pupation site. In our initial field survey, we quantified cocooning larvae on branches and trunks of trees. The typically smooth-bark branches in the lower, middle and upper canopy were nearly devoid of *C. pomonella* cocoons. Similarly, in two high-density planted orchards with smooth-bark trees, the three cocooned *C. pomonella* larvae that we located were in the duff layer of the soil at the base of trees, and no larvae were located within trees.

There is mounting evidence that aggregates of *C. pomonella* cocoons form, in part, in response to aggregation pheromone (Jumean et al., 2004, 2005a, b, 2007, 2008; this study). The probability of aggregates forming is likely directly proportional to the density of *C. pomonella* populations. At high population densities, many larvae may complete development on the same tree, and may aggregate in the same pupation site on its trunk. If, as proposed by Duthie et al. (2003), adults eclosing from aggregate cocoons more readily acquire mates and produce more offspring than their counterparts eclosing from solitary pupae, then aggregate cocoons aggravate the inefficiency of pheromone-based disorientation of adult males that is observed in orchards with high *C.*
pomonella population densities (Barclay, 1992; Barclay and Judd, 1995). Even at low to moderate population levels and otherwise effective population control measures, mated females may originate from aggregate cocoons, and their offspring may generate damaged fruit in localized “hot spots”.

With evidence that larval aggregations of C. pomonella can be frequent (this study), and that synthetic larval aggregation pheromone attracts fifth-instar larvae in the field (Jumean et al. 2007), an attract-and-kill formulation for larvae should be considered as a potential control tactic for C. pomonella in commercial apple orchards.

2.6 Acknowledgements

Thanks to Donald Thomson, Mike Doerr, Jay Brunner, and Alan Knight for assistance in locating suitable field sites, and Gary J.R. Judd for helpful comments on the manuscript. Financial support was provided by a Natural Sciences and Engineering Research Council of Canada (NSERC) – Canada Graduate Scholarship to ZJ and by a NSERC – Industrial Research Chair to G. Gries with Pherotech International Inc., SC Johnson Canada, and Global Forest Science as industrial sponsors.

2.7 Literature cited


Cydia pomonella larvae (Lepidoptera: Olethreutidae) produce and respond

Identification of the larval aggregation pheromone of codling moth, Cydia

parasitoids eavesdrop on cocoon-spinning codling moth, Cydia pomonella,

Pheromone-based trapping of larval codling moth, Cydia pomonella, in

of codling moth, Cydia pomonella, induce attraction or arrestment of

Jumean, Z., E. Jones, and G. Gries. 2009. Does aggregation behavior of
codling moth larvae, Cydia pomonella, increase the risk of parasitism by

Oxford, U.K.


Table 2.1 Descriptive statistics of *C. pomonella* larval aggregations as well as observations of their location on tree trunks above ground and their cardinal sector. An asterisk (*) denotes a significant difference between categories within an orchard.
### Descriptive statistics of aggregations

<table>
<thead>
<tr>
<th></th>
<th>Orchard 1</th>
<th>Orchard 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range of group size</td>
<td>2 – 20</td>
<td>2 – 16</td>
</tr>
<tr>
<td>Mean of group size</td>
<td>$3.33 \pm 0.17$</td>
<td>$3.48 \pm 0.19$</td>
</tr>
<tr>
<td>Mean sex ratio (male:female) in group</td>
<td>$1.08:1 \pm 0.04$</td>
<td>$1.04:1 \pm 0.03$</td>
</tr>
</tbody>
</table>

### Number of observations

Number of larval aggregations on tree trunks at specific height ranges above the soil line

<table>
<thead>
<tr>
<th>Height Range (cm)</th>
<th>Orchard 1</th>
<th>Orchard 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – 10 cm</td>
<td>9</td>
<td>21</td>
</tr>
<tr>
<td>11 – 20 cm</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td>21 – 30 cm</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>31 – 40 cm</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>41 – 50 cm</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>51 – 60 cm</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>61 – 70 cm</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>&gt; 70 cm</td>
<td>1</td>
<td>8</td>
</tr>
</tbody>
</table>

Cardinal direction of aggregation

<table>
<thead>
<tr>
<th>Cardinal Direction</th>
<th>Orchard 1</th>
<th>Orchard 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>North</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>South</td>
<td>17</td>
<td>37*</td>
</tr>
<tr>
<td>East</td>
<td>20</td>
<td>31</td>
</tr>
<tr>
<td>West</td>
<td>26</td>
<td>25</td>
</tr>
</tbody>
</table>
Figure 2.1  Frequency distribution of *C. pomonella* larval aggregations in Orchards 1 and 2. Shaded bars indicate the number of observations of a particular group size, whereas open bars indicate the number of specimens found in a group size. Total numbers of observations or specimens are given within bars. Bars with different letters within each observation group are significantly different by the $\chi^2$ goodness-of-fit test for multiple categories followed by subdivision of categories.
Figure 2.2  Number of *C. pomonella* larvae cooened singly or in aggregates of ≥2 specimens on tree trunks in Orchards 1 and 2. Total numbers of specimens cooening singly or in aggregates are given within bars. An asterisk (*) indicates a significant preference for a specific cooening behaviour; $P < 0.001$. 
3: IDENTIFICATION OF THE LARVAL AGGREGATION PHEROMONE OF CODLING MOTH, *Cydia pomonella*¹

3.1 Abstract

Mature larvae of the codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae), exit the fruit and seek sites suitable for pupation. Spinning cocoons in such sites, larvae produce a complex, cocoon-derived blend of volatiles recently shown to attract and/or arrest both conspecific larvae and the prepupal parasitoid *Mastrus ridibundus* Gravenhorst (Hymenoptera: Ichneumonidae). Here we report components of this blend that constitute the pheromone of fifth-instar *C. pomonella* larvae. Thirty-one two-choice olfactometer experiments showed that a blend of synthetic (E)-2-octenal, (E)-2-nonenal, sulcatone, and geranylacetone, in combination with either 3-carene and/or three saturated aldehydes (octanal, nonanal, decanal), elicited behavioural responses from *C. pomonella* larvae. In on-tree experiments with corrugated cardboard bands as pupation sites for larvae affixed to tree trunks, and with laboratory-reared larvae released onto such trees, more larvae cocooned in those halves of cardboard bands baited with cocoon-spinning conspecific larvae, or with synthetic pheromone components, than in unbaited control halves of the bands. With the larval aggregation pheromone identified in this study, there might be an opportunity to manipulate *C. pomonella* larvae in commercial fruit or nut orchards.
3.2 Introduction

When mature larvae of the codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae), complete their development, they exit the fruit and seek sites suitable for pupation. While spinning cocoons in such sites, larvae produce an aggregation pheromone that attracts or arrests conspecific larvae (Duthie et al., 2003; Jumean et al., 2004a). Aggregation of fifth-instars prior to pupation may be part of a mating strategy (Duthie et al., 2003) because in laboratory bioassays, eclosed adult males appeared to be arrested by the sex pheromone \( (E,E)\)-8,10-dodecadienol emanating from mature female pupae, which may allow mating as soon as a female ecloses.

Identification of the cocoon-derived pheromone proved challenging because larval antennae were too small to be used effectively in gas chromatographic-electroantennographic detection (GC-EAD) analyses of cocoon volatiles. Testing the hypothesis that *Mastrus ridibundus* Gravenhorst (Hymenoptera: Ichneumonidae), a parasitoid of late instar/prepupal *C. pomonella*, exploits odors produced by or associated with larvae as a kairomone during host-foraging, Jumean et al. (2004b) demonstrated (1) that 10 cocoon volatiles [3-carene, myrcene, heptanal, octanal, nonanal, decanal, (E)-2-octenal, (E)-2-nonenal, sulcatone, and geranylacetone] elicited responses from female *M. ridibundus* antennae, and (2) that eight of these components [all except myrcene and (E)-2-nonenal] were essential for the attraction of *M. ridibundus* in behavioural bioassays. A blend of the same 10 components and (+)-limonene
(an abundant compound in cocoon volatiles) as an 11th component also attracted/arrested foraging fifth-instar *C. pomonella* larvae (Jumean et al., 2004b). Here we report that a blend of (E)-2-octenal, (E)-2-nonenal, sulcatone, and geranylacetone in combination with either 3-carene and/or three saturated aldehydes (octanal, nonanal, decanal) elicited attraction or arrestment of pupation site-seeking fifth-instar *C. pomonella* larvae.

### 3.3 Materials and methods

#### 3.3.1 Experimental insects

Larvae were shipped in trays of artificial diet from the Sterile Insect Release Program rearing facility in Osoyoos, British Columbia, Canada. Trays containing 1000 larvae were kept in a glass aquarium (60×31×31 cm) and stored at 15°C under a 16L:8D photoperiod. Non-diapausing fifth-instar larvae were removed from the diet as needed for experiments.

#### 3.3.2 Acquisition of volatiles

To acquire naturally emitted volatiles for olfactometer experiments, 300 cocoon-spinning male and female fifth-instars (1:1 sex ratio) were placed in a cylindrical Pyrex glass chamber (15.5×20 cm). An empty chamber served as control. A water aspirator drew charcoal-filtered air at ~2 l/min through each chamber and through a glass column (14×1.3 cm OD) containing Porapak Q (50-80 mesh; Waters Associates, Inc., Milford, MA, USA). After 72 hr, volatiles were
eluted from the Porapak Q trap with 3 ml of pentane and ether (95:5). Extracts were concentrated under a nitrogen stream so that 1 ml was equivalent to 10 cocoon-spinning larvae-hour equivalents (10 CSLHE = volatiles released from 10 cocoon-spinning *C. pomonella* larvae during 1 hr). Extracts were stored in darkness at 15˚C, and analyzed by coupled gas chromatography-mass spectrometry (GC-MS) in full-scan electron impact mode, using a Varian Saturn 2000 Ion Trap GC-MS fitted with a DB-5 column (30 m×0.25 mm i.d., J&W Scientific, Folsom, CA, USA).

### 3.3.3 Olfactometer experiments

In two-choice Petri dish olfactometers (detailed drawing in Duthie et al., 2003), test stimuli were randomly assigned to one of two 4-ml vials (Table 3.1), each with a perforated Eppendorf tube to prevent physical contact of experimental larvae with test stimuli. Stimuli were pipetted onto Whatman no. 1 filter paper disks (1 cm diam.), with treatment and control disks receiving the same amount of solvent. For each replicate, one fifth-instar was placed in the center of the olfactometer, and whether it chose to cocoon in the treatment or control vial was recorded 18-24 hr later. All experiments were conducted at 21-26˚C in complete darkness.

To determine whether storage of cocoon volatile extract diminished its attractiveness, experiments 1 and 2 tested the responses of larvae to 180 CSLHE of fresh (1 d old) and aged (8 d old) extracts. In experiment 3, a synthetic blend (SB) of 11 cocoon volatiles [heptanal, octanal, nonanal, decanal, *(E)*-2-
octenal, (E)-2-nonenal, (+)-limonene, myrcene, 3-carene, sulcatone, geranylacetone] (Jumean et al., 2004b) was tested at 200 CSLHE to determine whether it had a comparable behavioural effect as Porapak Q extract of natural cocoon volatiles. Testing natural vs. synthetic cocoon volatiles or two blends of synthetic cocoon volatiles in the same confined olfactometer was attempted but found to compromise the larva’s ability to discriminate between volatile blends (Z. Jumean, unpublished data).

Taking the results of experiment 3 into account, experiments 4-7 tested SB at four doses (1, 10, 100, and 1000 CSLHE) to determine the optimal dose for subsequent experiments. To determine essential pheromone components, experiments 8-16 tested SB vs. blends lacking certain classes of organic chemicals (Byers, 1992), such as ketones (experiment 8), monoterpenes (experiment 9), or aldehydes (experiment 10). Experiments 11-16 took a similar approach by deleting from SB either saturated aldehydes (experiment 11), unsaturated aldehydes (experiment 12), or individual components (experiments 13-16). Considering the results of experiments 8-16, experiments 17-26 tested a four-component rudimentary synthetic blend (RSB) [(E)-2-nonenal, (E)-2-octenal, sulcatone, geranylacetone] alone (experiment 17) or in combination with one of three monoterpenes [(+)-limonene, myrcene, or 3-carene; experiments 18-20], or with one of four 3-component blends of saturated aldehydes [heptanal, octanal, nonanal, decanal; experiments 21-24]. Considering that only the blend of saturated aldehydes that contained octanal, nonanal, and decanal enhanced the effectiveness of RSB (experiment 22), experiment 25 explored whether decanal
could be deleted from this blend without affecting the blend’s behavioural activity. Experiment 26 then was designed to confirm that the RSB plus four essential components [3-carene (experiment 20); octanal, nonanal, and decanal (experiments 22, 25)] was attractive to larvae seeking pupation sites. Final laboratory experiments 27-31 explored whether SB at the low and behaviourally inactive dose of 10 CSLHE would become stimulatory upon increasing the amount of 3-carene (experiment 28), or either one or both of (E)-2-octenal and (E)-2-nonenal (experiments 28-31).

The on-tree experiments (32-36) were conducted at Simon Fraser University (May to October 2003) and employed 4-cm wide corrugated cardboard bands (cut from stock of 0.46-76 m single-face corrugated cardboard; Shippers Supply Inc., British Columbia, Canada). Cardboard bands were affixed with metal wire 45 cm above ground to trunks of maple (Acer spp.) trees that were 10-16 cm in diam at that height. Bands were divided into two halves, with test stimuli applied to the waxed center (4 cm²) of each half.

For each replicate in experiments 32-36, 20 fifth-instars were released from a thin circular collar affixed to the tree’s main branch crotch (~1.50 m above ground). Experiments were started at 22:00 hr and terminated 10-12 hr later by recording the number of larvae cocooning in treatment or control halves of the cardboard bands. Experiments 32 and 33 tested whether cardboard band halves baited with 25 1-d-old C. pomonella cocoons containing larvae or prepupae (experiment 32), or baited with a synthetic blend at 1000 CSLHE (experiment 33), attracted or arrested more C. pomonella larvae than did unbaited cardboard
band halves. In experiment 32, female larvae served as test stimuli and male larvae (as determined by testes visible through the dorsal integument) were bioassayed to allow recognition and recording of those larvae that had responded to test stimuli. This experimental design was justified because both male and female cocoon-spinning larvae produce and respond to the same volatile components (Jumean et al., 2004a). Experiments 34-35 determined whether a 10-fold decrease (experiment 34) or a 10-fold increase (experiment 35) in the dose of the synthetic blend affected the response of *C. pomonella* larvae in the field. Finally, experiment 36 explored whether the synthetic blend at the low and behaviourally inactive dose of 100 CSLHE (see experiment 34) would become active by increasing the amounts of (*E*)-2-octenal and (*E*)-2-nonenal as essential blend components.

### 3.3.4 Statistical analyses

Numbers of larvae responding to treatment and control stimuli in laboratory olfactometer experiments were analyzed with the $\chi^2$ goodness-of-fit test, using Yates correction for continuity ($\alpha = 0.05$) (Zar, 1999). Numbers of larvae responding to treatment and control stimuli in on-tree bioassays were analyzed with the Wilcoxon paired-sample test ($\alpha = 0.05$) (Zar, 1999).

### 3.4 Results

In laboratory olfactometer experiments, both fresh and aged Porapak extracts of cocoon volatiles at 180 CSLHE attracted larvae (Figure 3.1;
experiments 1, 2), as did a synthetic blend (SB) of 11 candidate pheromone components at 200 CSLHE (Figure 3.1, experiment 3). SB elicited a behavioural response also at 100 CSLHE (Figure 3.1; experiment 6), but not at 1, 10, or 1000 CSLHE (Figure 3.1; experiments 4, 5, 7). SBs lacking ketone or monoterpane components remained moderately attractive (Figure 3.2; experiments 8, 9), whereas an SB lacking aldehydes was inactive (Figure 3.2; experiment 10). An SB lacking saturated aldehydes was still active (Figure 3.2; experiment 11), whereas SBs lacking one or both unsaturated aldehydes [(E)-2-nonenal or (E)-2-octenal], or ketones [sulcatone or geranylacetone] elicited no significant responses from larvae (Figure 3.2; experiments 12-16). A rudimentary synthetic blend [RSB: (E)-2-octenal, (E)-2-nonenal, sulcatone, and geranylacetone] was not attractive (Figure 3.3; experiment 17), but the addition of 3-carene, unlike myrcene or (+)-limonene, rendered RSB attractive (Figure 3.3; experiments 18-20). Addition of the three saturated aldehydes octanal, nonanal, and decanal, unlike other three-component blends of saturated aldehydes, also rendered RSB attractive (Figure 3.3; experiments 21-24) to a level comparable with that of RSB plus all four saturated aldehydes (= SB minus monoterpenes; experiment 9). Addition of only octanal and nonanal to RSB failed to elicit a response from pupation site-seeking larvae (Figure 3.3; experiment 25), but RSB plus 3-carene, and octanal, nonanal, and decanal did elicit a behavioural response (Figure 3.3; experiment 26). SB at the low dose of 10 CSLHE had no effect on larval behaviour (Figure 3.4; experiment 27). Ten-fold increases of either 3-carene, (E)-2-octenal, or (E)-2-nonenal in that low-dose blend did not modify its
attractiveness (Figure 3.4; experiments 28-30) but a 10-fold increase of both (E)-2-octenal and (E)-2-nonenal in that blend stimulated a positive response from larvae (Figure 3.4; experiment 31).

In on-tree experiments, cocoons from conspecifics (experiment 32), and SB at 1000 CSLHE (experiment 33), attracted or arrested *C. pomonella* larvae foraging for pupation sites (Figure 3.5). In contrast, SB at 100 or 10,000 CSLHE was not active (Figure 3.5; experiments 34-35). Although the SB had no behavioural effect at 100 CSLHE (experiment 34), a 10-fold increase of both (E)-2-octenal and (E)-2-nonenal in that blend produced responses from larvae (experiment 36).

### 3.5 Discussion

Our laboratory and field data provide evidence that cocoon-spinning *C. pomonella* larvae produce a pheromone that attracts and/or arrests conspecific larvae seeking pupation sites (Duthie et al., 2003; Jumean et al., 2004a). The cocoon-derived 11 candidate pheromone components that were bioassayed in olfactometer experiments 1-31 were selected based on evidence that they attracted not only *M. ridibundus* parasitoids but also *C. pomonella* larvae (Jumean et al., 2004b). The comparable biological activity of the 11-component synthetic blend and the Porapak Q extract of cocoon volatiles (Figure 3.1, experiments 1-3) suggested that all essential pheromone components were present in the synthetic blend.
The aggregation pheromone of *C. pomonella* larvae is surprisingly complex and responses were critically dependent on dose and blend composition. Low- or high-dose blends were ineffective (Figure 3.1), as were blends lacking either (E)-2-octenal, (E)-2-nonenal, sulcatone, or geranylacetone (Figure 3.2; experiments 13-16). Synergism between components was also evident when the four-component rudimentary blend of (E)-2-octenal, (E)-2-nonenal, sulcatone, and geranylacetone failed to affect larval behaviour (Figure 3.3, experiment 17), but addition of either 3-carene (experiment 20) or three saturated aldehydes (octanal, nonanal, decanal) (experiment 23) resulted in attractive blends. Similar positive effects caused by 3-carene, or by saturated aldehydes, suggested redundancy in the blend composition. With five components needed for *C. pomonella* larvae to respond (Figure 3.3; experiment 20), and eight components needed for *M. ridibundus* parasitoids to respond (Jumean et al., 2004b), it appears that *M. ridibundus* requires a more complex signal to locate *C. pomonella* host prepupae than *C. pomonella* larvae require to communicate among themselves. The fact that the pheromone at low dose but with 10-fold increase of (E)-2-octenal and (E)-2-nonenal elicited a behavioural response from larvae (Figure 3.4; experiment 31) suggests that both of these unsaturated aldehydes are major components of the pheromone blend.

Pheromone components are perceived by *C. pomonella* larvae as airborne signals because baffles in olfactometer experiments prevented physical contact with natural or synthetic pheromone. However, whether the pheromone serves primarily to attract or arrest conspecific larvae is not yet known. The possible
adaptive significance of pheromone-based larval aggregation will be intriguing to investigate. Duthie et al. (2003) proposed that aggregations of *C. pomonella* larvae are part of a reproductive strategy that facilitates the earliest possible mating of eclosed adults. The proposed fitness advantage, however, may be offset by costs associated with larval aggregations. Host-derived pheromones are reliable indicators of host presence (Wiskerke et al., 1993; Wertheim et al., 2003), and are exploited by foraging parasitoids as illicit receivers of such signals (Stowe et al., 1995; Haynes and Yeargan, 1999; Jumean et al., 2004b). If, however, foraging parasitoids are egg-limited (Bezemer and Mills, 2001), individual *C. pomonella* larvae or prepupae in aggregations may be at a lower risk of parasitism than larvae that cocoon in isolation.

Aggregation of *C. pomonella* larvae as part of a proposed reproductive strategy (Duthie et al., 2003) might explain localized fruit damage in orchards treated with synthetic sex pheromone for *C. pomonella* control. Female *C. pomonella* eclosing from larval aggregations might be mated irrespective of otherwise functional pheromone-based tactics to disorient or attract and kill mate-foraging males. With the larval aggregation pheromone identified in this study, and shown to attract or arrest larvae in on-tree experiments (Figure 3.5; experiments 32-36), there may be a new opportunity to manipulate *C. pomonella* larvae in commercial fruit or nut orchards. Larval manipulation would be compatible with other biorational tactics of *C. pomonella* control including pheromone-mediated mating disruption and postharvest fruit removal (Judd et al., 1997).
3.6 Acknowledgements

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3.7 Literature cited


JUDD, G. J. R., GARDINER, M. G. T., and THOMSON, D. R. 1997. Control of


Table 3.1  Details on experimental insects and stimuli tested in laboratory
olfactometer and on-tree experiments.
Petri dish olfactometer bioassays

<table>
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<td>2</td>
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<tr>
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</tr>
<tr>
<td>4</td>
<td>1 SB</td>
<td>Solvent</td>
<td>30</td>
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<tr>
<td>5</td>
<td>10 SB</td>
<td>Solvent</td>
<td>28</td>
</tr>
<tr>
<td>6</td>
<td>100 SB</td>
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</tr>
<tr>
<td>7</td>
<td>1000 SB</td>
<td>Solvent</td>
<td>26</td>
</tr>
<tr>
<td>8</td>
<td>200 SB minus ketones</td>
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</tr>
<tr>
<td>9</td>
<td>200 SB minus monoterpenes</td>
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<td>16</td>
<td>200 SB minus geranylacetone</td>
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<td>200 Rudimentary Synthetic Blend(^d) (RSB)</td>
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18  200 RSB plus (+)-limonene  Solvent  20
19  200 RSB plus myrcene  Solvent  30
20  200 RSB plus 3-carene  Solvent  32
21  200 RSB plus heptanal plus octanal plus nonanal  Solvent  28
22  200 RSB plus octanal plus nonanal plus decanal  Solvent  27
23  200 RSB plus heptanal plus nonanal plus decanal  Solvent  23
24  200 RSB plus heptanal plus octanal plus decanal  Solvent  25
25  200 RSB plus octanal plus nonanal  Solvent  25
26  200 RSB plus 3-carene plus octanal plus nonanal  Solvent  30
   plus decanal
27  10 SB  Solvent  40
28  10 SB (3-carene 10-fold increased)  Solvent  33
29  10 SB [(E)-2-octenal 10-fold increased]  Solvent  47
30  10 SB [(E)-2-nonenal 10-fold increased]  Solvent  39
31  10 SB [(E)-2-octenal 10-fold increased plus  Solvent  36
    (E)-2-nonenal 10-fold increased]

On-tree experiments

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<tr>
<td>34</td>
<td>100 SB</td>
</tr>
<tr>
<td>35</td>
<td>10,000 SB</td>
</tr>
<tr>
<td>36</td>
<td>100 SB [(E)-2-octenal 10-fold increased plus (E)-2-nonenal 10-fold increased]</td>
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\(^a\) CSLHE = cocoon-spinning larvae hour equivalents.

\(^b\) Solvent consisted of redistilled pentane (20-100 ml).

\(^c\) 10 SB = synthetic blend of 11 components: decanal (1.4 ng) [Aldrich], nonanal (4.1 ng) [Aldrich], octanal (0.94 ng) [Aldrich], heptanal (0.85 ng) [Aldrich], (E)-2-nonenal (1.00 ng) [Bedoukian], (E)-2-octenal (0.41 ng) [Bedoukian], geranylacetone (0.50 ng) [Aldrich], sulcatone (0.81 ng) [Aldrich], 3-carene (0.95 ng) [Aldrich], myrcene (0.84 ng) [Aldrich], (+)-limonene (10.00 ng) [Aldrich].

\(^d\) 10 RSB = rudimentary synthetic blend of four components: (E)-2-nonenal (1.00 ng), (E)-2-octenal (0.41 ng), geranylacetone (0.50 ng), sulcatone (0.81 ng).

\(^e\) Fifth-instar larvae were allowed to cocoon in an open-fluted cardboard (CB) strip (4 cm²).

\(^f\) Female larvae were used as test stimuli, and male larvae were bioassayed to allow recognition and recording of all those larvae that had responded to test stimuli.
Figure 3.1  Response of fifth-instar *Cydia pomonella* larvae in Petri dish olfactometers to extracts of cocoon-derived volatiles and to a synthetic blend (SB) of 11 candidate pheromone components (experiments 1-3), or to varying doses of SB (experiments 4-7). Number of larvae responding to each stimulus is given within bars; number of larvae not responding in each experiment given within parentheses; asterisks indicate a significant response to a particular treatment; $\chi^2$ test with Yates correction for continuity; **P < 0.005; ***P < 0.001. Ten SB consisted of three monoterpenes [(+)-limonene (10.00 ng), 3-carene (0.95 ng), myrcene (0.84 ng)], four saturated aldehydes (heptanal, octanal, nonanal, decanal), two unsaturated aldehydes [(E)-2-octenal (0.41 ng), (E)-2-nonenal (1.00 ng)], and two ketones [sulcatone (0.81 ng), geranylacetone (0.50 ng)]. Cocoons were 1-3 d old at the time of aeration but Porapak Q extracts were tested before and after aging to determine stability of semiochemicals. Aliquots of 180 or 200 CSLHE (cocoon-spinning larvae hour equivalents) were tested in experiments 1-3. Aliquots of 1, 10, 100, or 1000 CSLHE were tested in experiments 4-7; the same amount (20-25 μl) of pentane was applied to treatment and control stimuli in experiments 1-7.
<table>
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<tr>
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<th>180 CSLHE (1-d-old)</th>
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**EXP. 2**

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**EXP. 3**

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**EXP. 4**

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**EXP. 5**

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**EXP. 6**

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**EXP. 7**

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<th>EXP. 7</th>
<th>1,000 SB</th>
<th>18 (14)</th>
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Figure 3.2  Response of fifth-instar *Cydia pomonella* larvae in Petri dish
olfactometers to synthetic blends (SB) lacking one or more
candidate pheromone components. Number of larvae responding to
each stimulus is given within bars; number of larvae not responding
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. Aliquots of 200 CSLHE (see caption of Figure 1)
were tested. The same amount (20 μl) of pentane was applied to
treatment and control stimuli.
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<th>Number of larvae responding</th>
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<td>(5) *</td>
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<td></td>
<td>Solvent control</td>
<td>9</td>
</tr>
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<td></td>
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<th>Number of larvae responding</th>
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<tr>
<td></td>
<td>200 SB minus aldehydes</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Solvent control</td>
<td>15</td>
</tr>
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<td></td>
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<tr>
<td></td>
<td>200 SB minus saturated</td>
<td>25</td>
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<tr>
<td></td>
<td>aldehydes</td>
<td>8</td>
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<tr>
<td></td>
<td>(7) *</td>
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<td>200 SB minus unsaturated</td>
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<tr>
<td></td>
<td>aldehydes</td>
<td>14</td>
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<td></td>
<td>200 SB minus (E)-2-octenal</td>
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<td>Solvent control</td>
<td>13</td>
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<td>12</td>
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<tr>
<td></td>
<td>Solvent control</td>
<td>18</td>
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<td>(10)</td>
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<td>Solvent control</td>
<td>13</td>
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<td>200 SB minus geranylacetone</td>
<td>19</td>
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<td></td>
<td>Solvent control</td>
<td>14</td>
</tr>
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<td></td>
<td>(7)</td>
<td></td>
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Figure 3.3  Response of fifth-instar *Cydia pomonella* larvae in Petri dish olfactometer experiments 17-26 to a rudimentary synthetic blend (RSB) of pheromone components and to RSB plus individual or groups of candidate pheromone components. Number of larvae responding to each stimulus is given within bars; number of larvae not responding given in parentheses; asterisks indicate a significant response to a particular treatment; $\chi^2$ test with Yates correction for continuity; *P < 0.01; **P < 0.005. Ten RSB consisted of two unsaturated aldehydes [(E)-2-octenal (0.41 ng), (E)-2-nonanal (1.00 ng)] and two ketones [sulcatone (0.81 ng), geranylacetone (0.50 ng)]. Aliquots of 200 CSLHE (see caption of Figure 1) were tested. The same amount (20 μl) of pentane was applied to treatment and control stimuli.
<table>
<thead>
<tr>
<th>Experiment</th>
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<th>Number of Larvae Responding</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
<td></td>
<td>Solvent control</td>
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</tr>
<tr>
<td>EXP. 18</td>
<td>200 RSB plus (+)-limonene</td>
<td>12 (20)</td>
</tr>
<tr>
<td></td>
<td>Solvent control</td>
<td>8</td>
</tr>
<tr>
<td>EXP. 19</td>
<td>200 RSB plus myrcene</td>
<td>18 (10)</td>
</tr>
<tr>
<td></td>
<td>Solvent control</td>
<td>12</td>
</tr>
<tr>
<td>EXP. 20</td>
<td>200 RSB plus 3-carene</td>
<td>25 (8)**</td>
</tr>
<tr>
<td></td>
<td>Solvent control</td>
<td>7</td>
</tr>
<tr>
<td>EXP. 21</td>
<td>200 RSB plus heptanal plus octanal plus nonanal</td>
<td>16 (12)</td>
</tr>
<tr>
<td></td>
<td>Solvent control</td>
<td>12</td>
</tr>
<tr>
<td>EXP. 22</td>
<td>200 RSB plus octanal plus nonanal plus decanal</td>
<td>21 (13)*</td>
</tr>
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<td>Solvent control</td>
<td>6</td>
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<td>EXP. 23</td>
<td>200 RSB plus heptanal plus nonanal plus decanal</td>
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</tr>
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<td></td>
<td>Solvent control</td>
<td>11</td>
</tr>
<tr>
<td>EXP. 24</td>
<td>200 RSB plus heptanal plus octanal plus decanal</td>
<td>16 (15)</td>
</tr>
<tr>
<td></td>
<td>Solvent control</td>
<td>9</td>
</tr>
<tr>
<td>EXP. 25</td>
<td>200 RSB plus octanal plus nonanal</td>
<td>14 (15)</td>
</tr>
<tr>
<td></td>
<td>Solvent control</td>
<td>11</td>
</tr>
<tr>
<td>EXP. 26</td>
<td>200 RSB plus 3-carene plus octanal plus nonanal plus decanal</td>
<td>24 (10)**</td>
</tr>
<tr>
<td></td>
<td>Solvent control</td>
<td>8</td>
</tr>
</tbody>
</table>
Figure 3.4  Response of fifth-instar *Cydia pomonella* larvae in Petri dish olfactometer experiments 27-31 to a synthetic blend (SB; see caption of Figure 1) of 11 components with the relative proportion of 3-carene or unsaturated aldehydes [(E)-2-octenal, (E)-2-nonenal] increased by 10-fold. Number of larvae responding to each stimulus is given within bars; number of larvae not responding given in parentheses; asterisks indicate a significant response to a particular treatment; $\chi^2$ test with Yates correction for continuity; *P < 0.05.

Aliquots of 10 CSLHE (see caption of Figure 1) were tested. The same amount (20 μl) of pentane was applied to treatment and control stimuli.
Figure 3.5  Response of male (experiment 32), or male and female (experiments 33-36), fifth-instar *Cydia pomonella* larvae in on-tree experiments to stimuli consisting of either 25 cocoons with female larvae (experiment 32), or synthetic blends (SB) of 11 components (see caption of Figure 1) at varying doses and component ratios. Strips of corrugated cardboard (CB) served as a pupation site. Number of larvae responding to each stimulus is given within bars; number of replicates given in parentheses; asterisks indicate a significant response to a particular treatment; Wilcoxon paired-sample test; *P < 0.01; **P < 0.005. Aliquots of 100, 1,000, or 10,000 CSLHE (see caption of Figure 1) were tested. The same amount (100 μl) of pentane was applied to treatment and control stimuli. In experiment 36 the proportion of (E)-2-octenal and (E)-2-nonenal in the blend was increased by 10-fold.
4: MALE AND FEMALE *Cydia pomonella* LARVAE

PRODUCE AND RESPOND TO AGGREGATION

PHEROMONE

---

4.1 Introduction

Insect aggregation pheromones are defined as chemical substances produced by members of one or both sexes that induce members of both sexes to form groups (Borden 1984). Aggregation pheromones are typically emitted by and attract adult insects. Interestingly, larvae of the codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae), seeking pupation sites aggregate in response to pheromone produced by cocoon-spinning conspecific larvae (Duthie et al. 2003). Such aggregations may be part of a mating strategy in which protandrous males are arrested by sex pheromone emanating from mature female pupae, thus allowing mating to ensue as soon as a female moth ecloses (Duthie et al. 2003). The cocoon-derived pheromone also attracts the specialist prepupal parasitoid *Mastrus ridibundus* Gravenhorst (Hymenoptera: Ichneumonidae), which “eavesdrops” on pheromonal communication of cocoon-spinning larvae (Jumean et al. 2005b).

Attraction of *M. ridibundus* is mediated by a blend of eight cocoon volatiles: heptanal, octanal, nonanal, decanal, (\(E\))-2-octenal, 3-carene, sulcatone, and geranylacetone (Jumean et al. 2005b). A very similar blend of cocoon volatiles — (\(E\))-2-octenal, (\(E\))-2-nonenal, sulcatone, and geranylacetone in combination with 3-carene and/or three saturated aldehydes (octanal, nonanal, and decanal) — was shown to attract or arrest mixed-sex *C. pomonella* larvae seeking pupation sites (Jumean et al. 2005a), indicating that the larval
pheromone is a blend of multiple components. However, no experimental study has yet determined (i) whether both male and female larvae produce and respond to the larval pheromone, and (ii) whether both sexes produce the same pheromone blend. Our objectives were to (i) determine the sex of *C. pomonella* larvae emitting and responding to the pheromone blend and (ii) quantify the amount of each volatile component produced by each sex. We hypothesize that both cocoon-spinning male and female larvae produce aggregation pheromone in equal quantities and concentrations.

### 4.2 Materials and methods

Codling moth larvae of various instars were shipped in trays of artificial diet from the Sterile Insect Release Program rearing facility in Osoyoos, British Columbia, Canada. This mass rearing has taken place since 1992 with several periodic introductions of feral specimens since this time. Trays were kept in a glass aquarium (60 cm × 31 cm × 31 cm) and stored at 15 °C and 16L:8D. Bioassay stimuli were generated by removing fifth-instar male (as determined by the presence of visible testes) or female larvae from the diet and allowing them to cocoon on strips (2.5 cm²) of corrugated cardboard. Empty cardboard strips served as control stimuli. Treatment and control stimuli were randomly assigned to one of two 4-mL vials in Petri dish olfactometers (Duthie *et al.* 2003). A perforated microcentrifuge tube in each vial prevented physical contact between bioassay insects and test stimuli (Fig. 4.1). For each replicate, one male or female fifth-instar larva was placed in the centre of the olfactometer and allowed
18-24 h to cocoon in either of the test vials. Any larvae cocooned outside of the vials were classified as non-responders. All experiments were conducted at 21–26 °C in complete darkness. Experiments tested whether male or female larvae were attracted to, or arrested by, five female prepupae in cocoons (experiments 1 and 2) or five male prepupae in cocoons (experiments 3 and 4), and whether they discriminated between five male and five female prepupae in cocoons (experiments 5 and 6). Whether responding larvae were attracted to, or arrested by, test stimuli has been tested in Chapter 5.

To acquire and analyze cocoon-derived volatiles, 200 cocoon-spinning male or female fifth-instar larvae were placed in a cylindrical Pyrex® glass chamber (9.0 cm × 16.5 cm). A water aspirator drew charcoal-filtered air at approximately 2 L/min through each chamber and through a glass column (140 mm × 1.3 mm o.d.) containing Porapak Q (50–80 mesh, Waters Associates, Inc, Milford, Massachusetts). After 72 h, volatiles were desorbed from Porapak Q with 3 mL of pentane. Extracts were concentrated under a nitrogen stream so that 1 μL was equivalent to 10 cocoon-spinning larvae hour equivalents (i.e., volatiles released from 10 cocoon-spinning *C. pomonella* larvae during 1 h).

Extracts were analyzed by coupled gas chromatography – mass spectrometry (GC–MS) in full-scan, electron-impact mode with a Varian Saturn Ion Trap GC–MS and a Hewlett-Packard 5985B GC–MS each fitted with a fused silica column (30 m × 0.25 or 0.32 mm i.d.) coated with DB-5 or DB-210 (J&W Scientific, Folsom, California).
4.3 Results and discussion

In olfactometer experiments, both male and female larvae were equally attracted to, or arrested by, volatiles from female (Fig. 4.2, experiments 1 and 2) and male cocoons (Fig. 4.2, experiments 3 and 4), with no preference for either volatile blend (Fig. 4.2, experiments 5 and 6). Both male and female larvae produced all components of the volatile blend (see above), with no quantitative differences in components between sexes (Table 4.1).

Identical volatile profiles produced by male and female cocoon-spinning larvae (Table 1), and similar responses of male and female larvae to such blends (Fig. 2), indicate that both male and female *C. pomonella* larvae produce and respond to an aggregation pheromone rather than a sex pheromone. These results are consistent with data obtained in orchard surveys that revealed a 1:1 sex ratio in *C. pomonella* larval aggregations (Jumean *et al.* 2009). Moreover, these field data confirm that both sexes respond to aggregation pheromone.

It will be intriguing to determine the adaptive significance of pheromone-based larval aggregation in *C. pomonella*. Potential benefits accrued by aggregating larvae include reduced risk of predation or parasitism by natural enemies (Wrorna and Dixon 1991, Low 2008) or expedient mate acquisition upon emergence from such an aggregation (Wertheim *et al.* 2002). It will also be interesting to investigate whether larvae of other insect species employ similar communication systems, as larval-mediated aggregation appears to be rare in holometabolous insects.
4.4 Acknowledgements

The research was financially supported by the Natural Sciences and Engineering Research Council of Canada through an Undergraduate Research Award to E. Rowland and a grant to G. Gries, and by a grant from the Washington Tree Fruit Research Commission to G.J.R. Judd and G. Gries.

4.5 Literature cited


| Table 4.1 | Comparative analysis of volatile components captured on Porapak Q from male and female fifth-instar, cocoon-spinning larvae of *Cydia pomonella*. |

*Cydia pomonella.*
Note: In each of six replicates for each sex, volatiles were acquired and analyzed from 200 cocoon spinning larvae. In 5 of 6 replicates, nonanal was the most abundant component. There were no quantitative differences in components between sexes (Student’s t-test, α = 0.05) (Zar 1999).

<table>
<thead>
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<th>Chemical component</th>
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<th>Female</th>
<th>P-value</th>
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<tr>
<td>3-carene</td>
<td>15.5±3.9</td>
<td>19.2±5.4</td>
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<tr>
<td>Octanal</td>
<td>19.4±1.3</td>
<td>16.8±1.3</td>
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<tr>
<td>Nonanal</td>
<td>98.0±2.1</td>
<td>92.9±7.1</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Decanal</td>
<td>29.0±6.0</td>
<td>18.0±2.6</td>
<td>&gt;0.05</td>
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<tr>
<td>(E)-2-octenal</td>
<td>5.0±0.9</td>
<td>4.4±0.7</td>
<td>&gt;0.05</td>
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<tr>
<td>(E)-2-nonenal</td>
<td>55.1±11.9</td>
<td>47.2±7.8</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Sulcatone</td>
<td>8.3±1.9</td>
<td>7.2±0.8</td>
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<tr>
<td>Geranylacetone</td>
<td>8.4±2.0</td>
<td>11.1±4.0</td>
<td>&gt;0.05</td>
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Figure 4.1  Schematic diagram of Petri-dish olfactometer.
Figure 4.2  Results of two-choice petri dish olfactometer (Duthie et al. 2003) experiments (Exp. 1–6) testing responses of fifth-instar male or female *Cydia pomonella* larvae to stimuli consisting of five cocoon-spinning male or female larvae. Strips of corrugated cardboard (CB) served as pupation sites for the test stimuli. Numbers of larvae responding to each stimulus are given within the bars and numbers of larvae not responding (i.e., cocooning outside vials that contained test stimuli) in each experiment are given in parentheses. Asterisks indicate a significant response to a particular treatment ($\chi^2$ test with Yates’ correction for continuity (Zar 1999); *, $P < 0.025$; **, $P < 0.01$; ***, $P < 0.001$).
<table>
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<th>Exp. 3</th>
<th>Exp. 4</th>
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<td>37</td>
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<td>30</td>
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<td>CB</td>
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<td></td>
</tr>
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<td>Cocoon + ♂ prepupa + CB</td>
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<td>28</td>
<td>(20)*</td>
<td>22</td>
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<td></td>
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</tr>
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<td></td>
<td>(23)</td>
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<td>(26)</td>
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<td>17</td>
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5: DOES LARVAL AGGREGATION PHEROMONE OF CODLING MOTH, *Cydia pomonella*, INDUCE ATTRACTION OR ARRESTMENT OF RECEIVERS?¹

5.1 Abstract

Cocoon-spinning larvae of the codling moth, *Cydia pomonella*, emit a pheromone that mediates aggregation by pupation site seeking fifth-instar larvae. It was unknown and thus we tested, whether the aggregation pheromone induces arrestment or attraction responses. In paired straight-tube experiment 1, fifth-instars moved faster and farther upwind toward cocooning conspecifics compared to blank controls. In still-air cage experiment 2, fifth-instars selected more often as first- and final choices of pupation sites those with cocooning conspecifics than those without. Finally, in Y-tube olfactometer experiment 3, fifth-instars anemotactically responded to, and preferred side arms with cocooning conspecifics to those without. Our data provide evidence that codling moth larvae are attracted to, rather than merely arrested by, larval aggregation pheromone. These results help explain reported aggregations or clumped distributions of larvae on tree trunks which would likely not occur if they were based merely on chance encounter of cocoon-spinning larvae by foraging larvae.

5.2 Introduction

Aggregation pheromones elicit behavioural responses in receivers which result in the clustering at the site of pheromone release. Receivers can be attracted to, or arrested by, aggregation pheromones. Arrestment responses are mediated by encounter of the pheromone source and are manifest in the
cessation of an animal’s locomotion through undirected kinetic responses, such as altered speed of locomotion (orthokinesis) or altered rate of turning (klinokinesis) (Fraenkel, 1961; Shorey, 1976; Wyatt, 2003). The concentration of arrestant pheromone affects the animal’s movements but not direction of movement (Shorey, 1976; Kennedy, 1978; 1986; Wyatt, 2003). Attractant aggregation pheromones, in contrast, attract receivers from a distance and induce oriented movements towards the pheromone source (Shorey, 1976; Wyatt, 2003). These “direct-guiding” responses, in which the receivers’ direction and orientation are related to the concentration gradient of the pheromone (Kennedy, 1986; Wyatt, 2003) can also be mediated by other stimuli, such as wind (anemotaxis) (Wyatt, 2003) or sound (phonotaxis) (Müller and Robert, 2001).

Larvae of the codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), are primary pests of apple and other pome fruits. Fifth-instar larvae exit the host fruit and seek concealed pupation sites in which to spin a cocoon and pupate. Foraging fifth-instar larvae aggregate in such sites due to aggregation pheromones emanating from the silk of cocoon-spinning conspecifics (Duthie *et al*., 2003; Jumean *et al*., 2004; 2005a, b; 2007).

The mechanisms of larval aggregation were unknown, and thus, we tested the hypothesis that foraging larvae are attracted to, rather than arrested by, aggregation pheromone. Specifically, we predicted that foraging larvae: 1) travel faster and farther in response to aggregation pheromone, and 2) orient to pheromone as a first-choice in both still-air and moving-air olfactometers.
5.3 Materials and methods

5.3.1 Experimental insects

All instar larvae were reared in artificial diet and shipped from the rearing facility of the Okanagan-Kootenay Sterile Insect Release Program (Osoyoos, BC, Canada). This mass rearing has taken place since 1992 with several introductions of feral specimens, the last of which occurring in 2006. Trays were stored in Rubbermaid™ containers (53 × 38 × 14 cm) and kept at 15˚C in complete darkness. Non-diapausing fifth-instar larvae were removed from the diet as needed and either deployed as a test stimulus or a bioassay insect. Larvae used as a test stimulus were allowed to cocoon 2-3 d in Petri dishes containing corrugated cardboard strips (2.5 cm² or 6 cm diam.) as pupation sites.

5.3.2 Olfactometer experiments

Experiment 1 tested both the velocity and distance a larva travelled in response to either a cardboard strip with 10 cocooned larvae (treatment) or an empty strip (control). In each replicate (n = 30), stimuli were randomly assigned to and inserted into the distal end of one of two straight Pyrex® glass tubes (40 × 2.3 cm i.d.) in parallel placement (Fig. 5.1A). One fifth-instar larva was gently placed at the mouth of each tube and aspirator-driven air was drawn through the tube at a rate of ~0.5 l/min. Both the distance a larva travelled upwind before reversing its orientation 180˚ or crossing a 30 cm marker line from the mouth of the tube and the time elapsed to travel this distance, were recorded.
Experiment 2 tested whether a larva orients towards aggregation pheromone as a first choice in still-air olfactometers. Test stimuli consisted either of a cardboard strip (6 cm diam.) harbouring 25 cocoons (treatment) or an empty strip (control) and were randomly assigned to and placed in opposite corners on the floor of a Plexi glass cage (30 × 30 × 40 cm). Each stimulus was surrounded by two electronic optical gates (9 × 1.5 × 1.5 cm) affixed to the floor of the cage (Fig. 5.1B). Each gate consisted of two opaque polyvinyl chloride blocks (1 × 1.5 × 1.5 cm) separated by a metal rod so that the blocks were held nine cm apart. Each block on each gate had mounted on it either a light-emitting diode (LED) (Stanley Electric Co. Ltd., CN305 880 nm, Deskin Sales, Vancouver, Canada) or its matching phototransistor (Stanley Electric Co. Ltd., PS302 peak sensitivity 800 nm, Deskin Sales, Vancouver, Canada) so that the optical centre was 0.3 cm above the cage floor. Each gate was connected via a four-wire cable to a programmable data logging device which recorded when the IR light beam was interrupted by a larva crossing the optical gate. The data-logging system, in turn, was connected to a Texas Instruments TravelMate™ 486 notebook computer which saved all recordings as a text file via a custom-made DOS-based program (Ray Holland, Science Technical Centre, Simon Fraser University, Burnaby, Canada). For each replicate, one fifth-instar larva was gently placed in the centre of the cage floor and allowed 24 h to select a pupation site. Crossing a gate by a larva in response to a stimulus was recorded by the computer and first and final choice of larvae were analyzed (n = 31 and n = 50, respectively). For a subset of
the replicates, the larva’s movement and first-choice of stimulus was observed for up to 2 h.

Experiment 3 tested anemotactic responses of larvae in Y-tube olfactometers (stem = 12 cm; arms = 19 cm; i.d. = 2.3 cm). Olfactometers were housed in a Styrofoam box (Fig 5.1C, modified from Derksen, 2006) with a light to dark gradient from the stem to the arms, encouraging a response from negatively phototactic larvae. Test stimuli consisted either of a cardboard strip (2.5 cm²) harbouring 10 cocooned larvae (treatment) or an empty strip (control), and were randomly assigned to, and placed within the distal 5 cm of the Y-tube arms. For each replicate (n = 21), a single fifth-instar larva was gently inserted into the stem of the tube and aspirator-driven air was drawn through the tube at a rate of 0.5 l/min. Larvae that penetrated >5 cm into either arm were classed as responders.

In experiments 1-3, all replicates were carried out at 20-23°C and ~50% R.H. Experiments 1 and 3 were conducted under red-light, whereas experiment 2 was conducted in complete darkness or under red-light to facilitate observations. For each replicate, a new fifth-instar larva was employed as a bioassay insect.

5.3.3 Statistical analyses

The mean distance and velocity that larvae travelled in experiment 1 were compared with a two-sample Student’s t-test. Numbers of larvae responding to stimuli in experiments 2 and 3 were analyzed with the $\chi^2$ goodness-of-fit test with
Yates correction for continuity. The experimental error rate in all experiments was set at $\alpha = 0.05$ (Zar, 1999).

5.4 Results

In experiment 1, fifth-instar larvae moved farther and faster in response to aggregation pheromone from cocooning larvae than in response to blank controls (Fig. 5.2). In experiment 2, fifth-instar larvae selected pupation sites with pheromone-emitting cocooning larvae as first and final choices significantly more often than empty pupation sites (Fig. 5.3). In experiment 3, significantly more fifth-instar larvae responded anemotactically to pupation sites with pheromone-emitting cocooning larvae than to empty pupation sites (Fig. 5.4).

5.5 Discussion

Farther and faster movement toward a pheromone source than toward a control (Fig. 5.2) indicated perception of pheromone over a distance of >30 cm, and suggested an attraction response. First-choice preference of foraging larvae for pupation sites with, rather than without, cocooning conspecifics (Fig. 5.3A) also implies orientation and directed movements towards the source of the aggregation pheromone, indicative of attraction responses. That the final-choice preference for pheromone-emitting sites was even more pronounced than the first-choice preference (Fig. 5.3B) can be attributed to some larvae crawling around, instead of through, the gates or crossing them without data-logging the
event. In the latter cases, first-choice preference of the larvae remained unknown.

Significantly stronger anemotactic responses by larvae in Y-tube olfactometers to pupation sites with cocooning conspecifics than to control sites (Fig. 5.4) demonstrated unambiguously that cocoon-spinning codling moth larvae emit aggregation pheromone that triggers attraction, not just arrestment, responses by foraging larvae.

The combination of chemo-orthokinetic responses or activating effects [i.e. increased speed (experiment 1)] coupled with oriented tactic responses (experiments 2 and 3)] results in attraction of the receivers toward attractive stimuli (Shorey, 1976; Kennedy, 1978). Based on our data, the active range of the pheromone appears to be at least 20 cm. It may not need to be greater because larvae seeking pupation sites are very mobile and repeatedly traverse the tree trunk. This displacement phase (Geier, 1963) is punctuated by an area-searching phase often when larvae encounter a trunk section with suitable pupation sites (Geier, 1963). In this area-searching phase, larvae typically stop and display characteristic head thrusts (Geier, 1963), possibly testing for the presence of aggregation pheromone at prospective pupation sites.

Our data clearly demonstrate that cocoon-spinning codling moth larvae produce an aggregation pheromone that triggers attraction responses by larvae seeking pupation sites. These results help explain reported aggregations or clumped distributions of larvae on tree trunks (Duthie et al., 2003; Z.J. personal
observation) which would not likely occur if they were based merely on chance encounter of cocoon-spinning larvae by foraging larvae.

5.6 Acknowledgements

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5.7 Literature cited


Figure 5.1 Set-up for experiments 1-3: A (1) test stimuli; (2) marker line 30 cm from mouth of tube; (3) 40 cm straight tube; (4) tube rack; (5) water-driven aspirator. B (1) test stimuli; (2) electronic optical gate; (3) gate-to-data logger cable; (4) data logger; (5) data logger-to-computer cable; (6) computer; (7) light-emitting diode or phototransistor. C (1) Styrofoam box; (2) Y-tube; (3) Nalgene® tubes; (4) 25-W light source 1 m below Y-tube stem; (5) activated-charcoal filter; (6) water-driven aspirator; (7) test stimulus; (8) red cellophane viewing window in Styrofoam box.
**Figure 5.2** Mean (± SEM) distance (A) and velocity (B) fifth-instar codling moth travelled upwind in paired straight-tube olfactometer experiment 1 towards a cardboard strip either with 10 codling moth larvae cocooned for 3 d (treatment) or empty (control). An asterisk (*) denotes treatments that are significantly different by Student's two sample t-test. Mean value is shown within bars for each treatment; n = 30 paired replicates.
Figure 5.3  First-choice (A) and final-choice (B) responses of fifth-instar codling moth larvae in cage experiment 2 to a cardboard strip either with 25 codling moth larval cocoons (3 d old) (treatment) or empty (control). An asterisk (*) denotes treatments that are significantly different by $\chi^2$ goodness-of-fit test using Yates correction for continuity. Numbers of larvae observed responding and total number of larvae responding are shown in white and hatched bars, respectively; n = 31 (A) and n = 50 (B).
A) 25 Cocoons: 8
Control: 6

Test stimulus: Number of larvae responding to conspecific cocoons or empty control cardboard strips as a first-choice

B) 25 Cocoons: 44
Control: 6

Test stimulus: Number of larvae responding to conspecific cocoons or empty control cardboard strips as a final choice
Figure 5.4 Anemotactic response of fifth-instar codling moth in Y-tube olfactometer experiment 3 to a cardboard strip either with 10 codling moth larvae cocooned for 3 d (treatment) or empty (control). An asterisk (*) denotes treatments that are significantly different by $\chi^2$ goodness-of-fit test using Yates correction for continuity. Numbers of larvae responding to each stimulus is shown within bars; n = 21.
6: DOES AGGREGATION BEHAVIOUR OF CODLING MOTH LARVAE, *Cydia pomonella*, INCREASE THE RISK OF PARASITISM BY *Mastrus ridibundus*?¹

¹ Published as: Jumean, Z., Jones, E., and Gries, G. 2009. Does aggregation behavior of codling moth larvae, *Cydia pomonella*, increase the risk of parasitism by *Mastrus ridibundus*? Biological Control. 49: 254-258.
6.1 Abstract

*Mastrus ridibundus* is a specialist hymenopteran parasitoid that parasitizes last-instar larvae or prepupae of the codling moth, *Cydia pomonella*. Foraging females eavesdrop on an aggregation pheromone produced by cocooning larvae. We investigated whether larvae that cocoon in aggregation experience a greater rate of parasitism than larvae that cocoon in isolation. In wind tunnel experiments, 10 larvae in aggregations were more readily located by female *M. ridibundus* than 10 larvae well separated from each other. Similarly, aggregations of 30 larvae were more attractive to female *M. ridibundus* than those of 3 larvae. In cage experiments, larval cocooning in aggregation or isolation had no effect on the mean rate of parasitism and the mean number of eggs deposited per parasitized host. In Petri-dish experiments, the location of larvae within an aggregation significantly affected their rate of parasitism, with those in the centre of an aggregation completely shielded from parasitism. Our data suggest that aggregation behaviour by *C. pomonella* larvae does not appear to increase the rate of parasitism. The increased risk of aggregated larvae being detected by *M. ridibundus* is likely offset by diluted parasitism risk and structural refugia effects that larvae in aggregation experience. As an egg-limited parasitoid, female *M. ridibundus* can parasitize on average only one larva in an aggregation, with the likelihood of parasitism for each larva being inversely proportional to the number of larvae in that aggregation.
6.2 Introduction

Larvae of parasitoid insects feed and develop in or on other host organisms, most commonly other insects, resulting in the death of the host (Godfray, 1994). Foraging female parasitoids utilize various cues from the host habitat or the hosts to locate them. Such cues may have visual, physical, or semiochemical characteristics (Vinson, 1998; DeLury et al., 1999).

Semiochemicals from host habitats are readily detectable by foraging parasitoids but are not reliable indicators of host presence. Semiochemicals from the host, in contrast, reliably indicate host presence, but often are not very abundant (Vet and Dicke, 1992; Stowe et al., 1995; Vinson, 1998). This presents a challenge for foraging parasitoids because selection favours host insects that remain as inconspicuous as possible to natural enemies. Host insects, however, may produce semiochemicals, such as trail, marking or aggregation pheromones, which serve functional roles in their biology (Hoffmeister et al., 2000; Wertheim et al., 2003) and thus undergo selection to remain part of the insect’s life history traits. Such semiochemicals are exploitable by natural enemies as dependable and reliable indicators of host presence. Moreover, parasitoids that “eavesdrop” on aggregation pheromones of their hosts may be at a particular advantage because aggregation pheromones are readily detectable cues during host foraging (Wertheim et al., 2003; Jumean et al., 2005b).

Larvae of the codling moth, Cydia pomonella L. (Lepidoptera: Tortricidae), develop in apples and other pome fruits. Mature larvae exit the fruit and pupate in
aggregations in cracks and crevices of host tree trunks (Duthie et al., 2003; Jumean et al., 2007). Aggregation is mediated by an 8-component pheromone that emanates from the silk of freshly spun cocoons (Jumean et al., 2005a,b). Both male and female larvae produce the pheromone (Jumean et al., 2004) and respond to it over a > 20 cm distance (Jumean et al., 2008). Proposed benefits of pheromone-based larval aggregations include efficient location of suitable pupation sites, expedient mating of eclosing adults, and reduced risk of parasitism.

*Mastrus ridibundus* (Gravenhorst) (Hymenoptera: Ichneumonidae) is a specialist ectoparasitoid of cocooned last-instar or pre-pupal *C. pomonella*. Synovigenic female parasitoids emerge with a complement of ~5 eggs, and after oviposition require >12 h to mature new eggs (Bezemer and Mills, 2001). *Mastrus ridibundus* was introduced to the United States as a biological control agent for *C. pomonella* (Unruh, 1997). Foraging female parasitoids eavesdrop on the communication of *C. pomonella* by utilizing the larval aggregation pheromone as a host-location kairomone. The kairomone reliably indicates the presence of *C. pomonella* larvae and attracts *M. ridibundus* from a distance. It continues to emanate from cocoons for several days after cocoon-spinning commences (Jumean et al., 2005b).

Here, we report the effect of larval aggregation on rate of parasitism by *M. ridibundus*. We predicted that (i) large aggregations of larvae emanate more aggregation pheromone and thus are more apparent to *M. ridibundus* than small aggregations or larvae in isolation; (ii) larvae in an aggregation do not suffer a
higher rate of parasitism than those in isolation because female *M. ridibundus* are egg-limited and can parasitize only few hosts within 12 hours; and (iii) larval aggregations are structural refugia that physically shield specimens in the centre from parasitism by *M. ridibundus*.

6.3 Materials and methods

6.3.1 Experimental insects

6.3.1.1 *Mastrus ridibundus*

*Mastrus ridibundus* parasitoids were originally collected in Kazakhstan (Unruh, 1997) and since 1994 reared at the USDA-Agricultural Research Station, Wapato, WA, USA. Overwintering *C. pomonella* host pupae parasitized with *M. ridibundus* were shipped to Simon Fraser University (SFU) in corrugated cardboard rolls that were kept in Plexiglas cages (40 × 30 × 30 cm) at 25-29°C, 30-50% R.H., and a photoperiod of 16L:8D in SFU’s Global Forest Quarantine Facility. Adult *M. ridibundus* were sustained with a 10% honey-water solution and a water-soaked cotton wick *ad libitum*. All parasitoids used in experiments were 3-14 days old and were isolated in 30-ml plastic cups provisioned with honey-water-soaked wicks (2 × 1 cm diam.) 18 h prior to the onset of experiments. Each insect was bioassayed once.
6.3.1.2 *Cydia pomonella*

Trays containing ~1000 fourth-instar *C. pomonella* were obtained from the Sterile Insect Release Program Facility, Osoyoos, BC, Canada and kept in a Rubbermaid™ container (53 × 38 × 14 cm) at 15˚C, 50% R.H., and a photoperiod of 16L:8D in SFU’s insectary.

6.3.2 Test stimuli for experiments

Effects of aggregation behaviour by *C. pomonella* larvae on oviposition behaviour by *M. ridibundus* were determined in laboratory bioassays by experimentally generating aggregations of various sizes as test stimuli.

Test stimuli for experiments 1-3 were generated by removing 1, 3, 10 or 30 fifth-instar larvae from trays, and allowing them to cocoon on corrugated cardboard discs (2.5 or 6 cm diam.) inside separate Petri dishes. After 3 d, discs to be tested in experiments 1-3 were enclosed in mesh to standardize visual cues and affixed to a single-faced cardboard cylinder (40 × 17 cm diam.) with corrugations facing outward (Fig. 6.1A, B).

To generate stacked larval aggregations for experiment 4, two single-faced cardboard discs (6 cm diam. each) were affixed to one another, corrugations facing out (Fig. 6.1C), and placed into a Petri dish (14 cm diam.). Twenty larvae were introduced to the shelter. Larvae that cocooned early in the discs’ seam were concealed by those that cocooned later and that were thus
exposed to the opening of the shelter. Three days later, the resulting stacked aggregations were subjected to parasitism by *M. ridibundus*.

6.3.3 Experimental protocol

6.3.3.1 Objective of experiment 1: To test whether *C. pomonella* larvae cocooning in aggregations are more apparent to foraging *M. ridibundus* than larvae cocooning in isolation.

In experiment 1 (n = 8), upwind (8 cm/s) flight by *M. ridibundus* was tested in a Plexiglas wind tunnel (1.10 × 1.10 × 3.30 m long) illuminated with diffused, broad-spectrum 40-W fluorescent light. Test stimuli consisted of two cardboard cylinders 1 m apart from each other and baited with one disc (6 cm diam.) carrying 10 cocooning *C. pomonella* larvae or 10 widely-spaced discs (2.5 cm diam.), each carrying a single cocooning larva (Fig. 6.1A, B). In each of 8 replicates, 5 female wasps were released from a Plexiglas box (10 × 10 × 6 cm) 2 m downwind from test stimuli, and the number of contacts parasitoids made with either stimulus was recorded for 2 h. For each replicate, the number of contacts with either stimulus was averaged over the 5 parasitoids, and data of all replicates were analyzed statistically (see below).
6.3.3.2 **Objective of experiment 2: To test whether C. pomonella larvae cocooning in large aggregations are more apparent to M. ridibundus than those in small aggregations**

In experiment 2 (n = 8), we tested if high concentrations of larvae are more apparent to *M. ridibundus* than low concentrations of larvae. To test this, we kept the experimental design and protocol identical to those in experiment 1 except that cardboard cylinders were baited with a single cardboard disc (6 cm diam.) that carried 3 or 30 cocooning *C. pomonella* larvae. These numbers representing small or large aggregations were based on sampling surveys in unmanaged apple orchards, revealing aggregations of 2-20 larvae (Z.J. *et al.*, unpublished data).

6.3.3.3 **Objective of experiment 3: To test whether varying cocooning concentrations of C. pomonella larvae affect the rate of parasitism (number of hosts parasitized during 12 h) or the number of eggs deposited per parasitized host.**

In experiment 3 (n = 12), a single female parasitoid was released into a Plexiglas cage (30 × 30 × 40 cm) illuminated with diffused, broad-spectrum 40-W fluorescent light. The cage contained a single corrugated cardboard cylinder to which were affixed either 10 widely separated discs (2.5 cm diam.) each carrying a single *C. pomonella* larva (Fig. 6.1A), or a single cardboard disc (6 cm diam.) carrying 10 cocooning *C. pomonella* larvae (Fig. 6.1B). After 12 h before the female parasitoid would have replenished her depleted complement of eggs
(Bezemer and Mills, 2001), she was removed from the cage and both the number of larvae parasitized and the number of eggs oviposited per parasitized larva were determined.

6.3.3.4 **Objective of experiment 4: To test whether the micro-location of cocooning larvae within aggregations affects their rate of parasitism.**

In experiment 4 (n = 10), one female parasitoid was released into a Petri dish (14 cm) that was illuminated with a diffused, broad-spectrum 40-W fluorescent light, and contained a cardboard shelter (Fig. 6.1C) with stacked cocooned larvae. Eight hours later, the female was removed and the number of larvae parasitized in the center and perimeter of the aggregation was recorded.

6.3.4 **Statistical analyses**

In experiments 1 and 2, the number of contacts female *M. ridibundus* made with *C. pomonella* larvae was arcsine-transformed. The mean number of (i) contacts female parasitoids made with each stimulus in experiments 1 and 2, (ii) hosts attacked and proportion of eggs oviposited by *M. ridibundus* in experiment 3, or (iii) proportion of larvae parasitized in the centre and perimeter of aggregations in experiment 4 were compared using a two-sample Student’s *t*-test. The experimental error rate in all experiments was set at $\alpha = 0.05$ (Zar, 1999).
6.4 Results

In experiment 1, host-foraging female *M. ridibundus* contacted cardboard cylinders with an aggregation of 10 cocooned *C. pomonella* larvae significantly more often than cylinders with 10 single and randomly distributed larvae on a cardboard cylinder (Fig. 6.2, Experiment 1; \( t = 3.96, \text{df} = 14, P < 0.001 \)). In experiment 2, female *M. ridibundus* contacted cylinders with an aggregation of 30 larvae significantly more often than cylinders with an aggregation of 3 larvae (Fig. 6.2, Experiment 2; \( t = 3.32, \text{df} = 14, P < 0.005 \)). In experiment 3, the 10 *C. pomonella* larvae that had cocooned in aggregation or in isolation suffered similar rates of parasitism by *M. ridibundus* over the 12-h oviposition period (Fig. 6.3, top; \( t = 0.84, \text{df} = 22, P > 0.05 \)), and parasitized host larvae received similar numbers of *M. ridibundus* eggs (Fig. 6.3, bottom; \( t = 0.23, \text{df} = 22, P > 0.05 \)). In experiment 4, *C. pomonella* larvae that had cocooned in the perimeter of an aggregation were parasitized significantly more often than larvae that had cocooned in the centre (Fig. 6.4; \( t = 3.54, \text{df} = 18, P < 0.005 \)).

6.5 Discussion

Pheromone-based aggregations of *C. pomonella* larvae represent a trade-off between potential benefits and costs of such behaviour. Benefits may include, but are not limited to, efficient location of suitable pupation sites and expedient mating of eclosing adults, whereas costs may be greater apparenty of aggregating larvae to foraging parasitoids or other natural enemies that eavesdrop on the larval communication. We investigated whether, and to what
extent, aggregations affect the risk of parasitism by the specialist parasitoid *M. ridibundus*. Our results suggest that the larval aggregation pheromone is an important kairomone that mediates long-range orientation by *M. ridibundus* toward prospective hosts. Cardboard cylinders with larval aggregations were frequented by female *M. ridibundus* more often than cylinders carrying the same number of widely-spaced single larvae (Fig. 6.2). Similarly, large aggregations were more apparent to *M. ridibundus* than small aggregations, likely because they emanate more kairomone. Thus, female *M. ridibundus* appear to exploit larval pheromone as a highly reliable indicator of host presence (Vinson, 1998) and proper developmental stage for parasitism (Jumean et al., 2005b). Our results are consistent with those of Bezemer and Mills (2001) who showed that female *M. ridibundus* forage for hosts more often on trees with high rather than low *C. pomonella* larval densities.

At high population densities, larvae may occur frequently in aggregations which, in turn, are more apparent to parasitoids and other natural enemies and more readily reveal patch profitability, allowing parasitoids to efficiently forage in high-yield patches (Hassell, 2000; Wertheim et al., 2003). In the parasitoid-host system of *Leptopilina heterotoma* and *Drosophila melanogaster* (Wertheim et al., 2003), the response of *L. heterotoma* is directly proportional to the amount of aggregation pheromone deposited by female *D. melanogaster* during oviposition. Substrates treated with high doses of *D. melanogaster* aggregation pheromone attracted more parasitoids than substrates with little or no aggregation pheromone (Wertheim et al., 2003). Similarly, Low (2008) has shown that mining
galleries produced by larvae of the leaf-mining moth _Antispila nysaefoliella_ are highly conspicuous to foraging natural enemies. Moreover, aggregated leaf mines increase the rate of host detection by parasitic wasps that specialize on the larvae.

Female _M. ridibundus_ appear to locate a host patch more readily when _C. pomonella_ larvae are in aggregation rather than isolation. However, once in a patch the rate of parasitism (Fig. 6.3, top), or the number of eggs deposited per parasitized host (Fig. 6.3, bottom), did not differ irrespective of the spatial pattern of cocooned larvae. Our results, and those by Bezemer and Mills (2001) showing that only one of ten hosts is parasitized, can be explained by life history traits of female _M. ridibundus_. Females are synovigenic, commencing egg production after eclosion with up to ~5 eggs per day (Bezemer and Mills, 2003). Considering that a female oviposits 2-7 eggs per host (Z.J., personal observation), and requires >12 h to generate a new complement of eggs, within 12 h of oviposition she will have eggs for just one bout of parasitism. Thereafter, she will likely leave the patch in search for new ones (Bezemer and Mills, 2001).

Even though _C. pomonella_ larvae in aggregations are more readily located by female _M. ridibundus_ (Fig. 6.2), these larvae also accrue benefits from such behaviour. With an egg-limited parasitoid that can only parasitize one larva per aggregation, a larva’s likelihood of parasitism is inversely proportional to aggregation size. Similar results were obtained by Low (2008) who showed that, despite increased detectability by parasitic wasps, _A. nysaefoliella_ larvae that forage in groups experience an increase in per capita survival as a result of
dilution effects. Therefore, the costs of increased detectability will likely be offset by dilution effects and thus survival advantages will select for larger aggregations (Low 2008). A similar inverse relationship between host detectability and parasitism rate has been shown for larvae of the arctiid moth *Ammalo helops* and its suite of parasitoids (Horgan, 2005).

The likelihood of parasitism is also affected by a larva’s micro-location within an aggregation. *Cydia pomonella* larvae in the center rather than perimeter of an aggregation are protected from parasitism. The ovipositor of female *M. ridibundus* is not sufficiently long to reach larvae in the center. Similar results have been reported for other insects. Larvae of the tephritid fruit fly *Urophora cardui* cause gall formation in plant tissue, with gall size affecting the likelihood of parasitism by *Eurytoma* spp. parasitoids that cannot reach larvae in large galls with their ovipositor (Zwölfer and Arnold-Reinhart, 1993). Furthermore, eggs of the reduviid bug *Psammolestes arthuri* are deposited in layers, with those on the perimeter experiencing higher rates of parasitism than those in the center of the egg mass (Feliciangeli, 1978).

In conclusion, aggregation behaviour by *C. pomonella* larvae does not appear to increase the risk of parasitism by female *M. ridibundus*. Ten larvae in aggregation were only twice as likely to be located by female *M. ridibundus* than 10 larvae well separated from one another (Fig. 6.2). This increased risk of detection by *M. ridibundus* is offset by diluted parasitism risk and structural refugia effects larvae in aggregation experience.
6.6 Acknowledgements

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6.7 Literature cited


Figure 6.1  Graphical illustrations of (A, B) corrugated cardboard cylinders to which were affixed discs with *Cydia pomonella* larvae that had cocooned singly or in aggregations of 3 or 30, and (C) a corrugated cardboard shelter to study parasitism rates of larvae in the centre and perimeter of larval aggregations within the seam of two cardboard discs affixed to one another.
Figure 6.2  Mean (± SE) number of contacts female *Mastrus ridibundus* made with corrugated cardboard cylinders to which were affixed (a) ten *Cydia pomonella* larvae cocooned in aggregation or isolation (experiment 1), and (b) three or ten larvae in aggregation (experiment 2). An asterisk (*) indicates a significant preference for a particular test stimulus; Student’s *t*-test, *P* < 0.05.
Figure 6.3  Mean (± SE) percent of *Cydia pomonella* host larvae parasitized by female *Mastrus ridibundus* and number of eggs deposited per parasitized host that had cocooned in aggregation or isolation in experiment 3.
Experiment 3

Test stimulus | Mean percent (± SEM) of C. pomonella larvae attacked by M. ridibundus

- Aggregated larvae: 15.0%
- Randomly distributed larvae: 10.8%

Test stimulus | Mean (± SEM) number of eggs deposited by M. ridibundus per parasitized C. pomonella host larva

- Aggregated larvae: 3.9
- Randomly distributed larvae: 4.3
**Figure 6.4** Mean (± SE) percent of *Cydia pomonella* larvae that were parasitized by *Mastrus ridibundus* based on their location in the perimeter or centre of an aggregation. An asterisk (*) indicates a location that had significantly higher parasitism; Student’s *t*-test, (*P* < 0.001).
Experiment 4

<table>
<thead>
<tr>
<th>Test stimulus</th>
<th>Mean percent (± SEM) of C. pomonella host larvae parasitized by M. ridibundus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perimeter hosts</td>
<td>14</td>
</tr>
<tr>
<td>Central hosts</td>
<td>0</td>
</tr>
</tbody>
</table>

*
7: A THEORETICAL APPROACH TO STUDY THE EVOLUTION OF AGGREGATION BEHAVIOUR BY CODLING MOTH LARVAE, CYDIA POMONELLA

1 Prepared for peer-review submission to Journal of Insect Behavior as: Jumean, Z., Ma, B.O., Chubaty, A.M., Ellenor, C.W., Roitberg, B.D., and Gries, G. 2010. A theoretical approach to study the evolution of aggregation behaviour by codling moth larvae.
7.1 Abstract

Pupation site-seeking larvae of the codling moth, *Cydia pomonella*, aggregate in response to aggregation pheromone produced by cocoon-spinning conspecific larvae. Larvae that cocoon in an aggregation rather than in solitude may experience a lower rate of parasitism by the parasitoid *Mastrus ridibundus*. Additionally, adults eclosing from a larval aggregation may encounter mates more rapidly at the site of eclosion (on-site) than away from that site (off-site).

We employed an evolutionary simulation to determine the effect of several ecological parameters on the evolution of larval aggregation behaviour. These parameters included (i) the probability of mate encounter offsite; (ii) the time available for finding a mate; and (iii) the population density of parasitoids and their rate of larval parasitism. The model predicts that larval aggregation behaviour is selected for when the probability of off-site mate encounter is low, the time to locate mates is short, and egg-limited parasitoids are at high population levels. We also show that aggregations might reduce the risk of parasitism through dilution effects. The parameters found to favour the evolution of larval aggregation behaviour are consistent with life-history traits exhibited by *C. pomonella*. 
7.2 Introduction

Group formation in animals generally requires the proximity of individuals in time and space. In numerous taxa of non-social species, individuals may join conspecifics (aggregate) in response to stimuli (“invitation” effects) from conspecifics. A joiner’s response to an invitation should evolve only when both the inviter and invitee accrue fitness benefits (Hamilton 1971; Prokopy and Roitberg 2001). Individuals may also join conspecifics in response to resource-derived cues, resulting in the formation of a group or an aggregation (Prokopy and Roitberg 2001; Krause and Ruxton 2002). Benefits conferred to aggregating individuals might include anti-predator effects (e.g., many-eyes effect, dilution effect, predator confusion), increased foraging ability (e.g., expedient resource location, improved resource extraction), increased probability of finding mates, reduced probability of desiccation, or improved ability to overcome plant defenses (Turner and Pitcher 1986; Krause and Ruxton 2002).

Insects aggregate in response to stimuli associated with a resource (e.g., food, mates, oviposition site, shelter) or produced by conspecifics (e.g., sex or aggregation pheromones). Individuals responding to such cues or signals gain information about the state of a resource, allowing them to gauge the profitability of joining or avoiding conspecifics that occupy it (Edgerly et al. 1998; Prokopy and Roitberg 2001). Pheromones mediate aggregations of adults in many non-social insects. However pheromonal communication between larvae of holometabolous insects is not common and known to occur only in a few species. Moreover, the costs and benefits of these larval interactions have hardly been
studied. Larvae of the Indian meal moth, *Plodia interpunctella*, produce a pheromone that induces oviposition by females, and thus increases the density of larval populations (Phillips and Strand 1994). This, in turn, facilitates nutrient extraction, reduces microbial growth in the resource, and improves protection from natural enemies through a large matting of larval silk (Phillips and Strand 1994; Prokopy and Roitberg 2001). At high larval densities and thus high concentrations of pheromone, the pheromone repels larvae, thereby regulating population densities of larvae at a food resource (Phillips and Strand 1994).

Larvae of the great spruce bark beetle, *Dendroctonus micans*, produce an aggregation pheromone and feed in groups rather than in individual tunnels, resulting in an increased growth rate (Deneubourg *et al.*, 1990; Storer *et al.* 1997).

Cocoon-spinning fifth-instar larvae of the codling moth, *Cydia pomonella*, produce and respond to an aggregation pheromone (Jumean *et al.* 2004, 2008). Larval aggregations may benefit both larvae and adults. Larvae experience a reduced risk of parasitism by the parasitoid *Mastrus ridibundus* (Jumean *et al.* 2009a) and eclosing adults are likely to immediately encounter mates. Rapid mating is advantageous because females experience a linear decrease in reproductive rate (fecundity and fertility) over time, with a 50% reduction four days after eclosion (Vickers 1997). Males eclosing near a female pupa are arrested by it significantly longer than males that eclose near a male pupa or no pupa (Duthie *et al.* 2003). This arrestment is due to sex pheromone of female moths that emanates from mature pupae prior to eclosion.
Despite the benefits of larval aggregations, there may also be associated costs. As aggregations increase in size they may become conspicuous visually or semiochemically to *M. ridibundus* (Jumean *et al.* 2009a) or other natural enemies that engage in area-restricted searches and could inflict significant mortality when they locate an aggregation.

Because aggregations of various sizes may have differential payoffs, a larva’s decision to join an aggregation also depends on cocooning decisions of conspecific larvae. As such, the decision whether or not to join an aggregation is circumstance-dependent and becomes a game theory question. It hinges upon the frequency of behaviours expressed by conspecifics. For example, a larva foraging together with other larvae must take into account their aggregation strategy. The costs and benefits of decisions to join large or small aggregations, or to cocoon singly, should depend on specific environmental conditions under which they accrue. Choosing an aggregation strategy that provides the greatest payoff requires adaptable decisions by a larva as conspecifics adapt and environmental conditions change.

Here we attempt to infer the evolutionarily stable (Maynard Smith 1982) aggregation behaviour of codling moth larvae under various ecological pressures, taking into account both larval and subsequent adult behaviours. Because the strategy of a larva is dependent on both the frequency of those choosing it and the abundance of larvae in the environment, we chose to employ a game theoretical individual-based simulation search algorithm model, in particular, a genetic algorithm (GA) to evaluate the effects of various ecological parameters.
on patterns of larval aggregation behaviour. GAs are inspired by the process of natural selection (Perry and Roitberg 2005). They are able to compete a large array of strategies against one another in a manner analogous to natural selection to find the optimal strategy or evolutionary stable strategies (Goldberg 1989; Forrest 1993). In GAs, each individual represents a specific strategy with no explicit genetics.

In our model we used the ‘phenotypic gambit’ approach (Grafen 1984), essentially ignoring any genetic constraints. Model parameters included the probability that an eclosed moth mates away from the pupation/aggregation site (off-site), the effects of parasitoid density and attack rate, and the time allocated to locate a mate after adult eclosion. We also examined the effect of localised (on-site) mating on the evolution of larval aggregation behaviour. We predicted that cocooning in aggregation rather than in solitude should be the dominant strategy in a population when (i) the probability of mating off-site is lower than mating on-site; (ii) aggregation behaviour mitigates pressure from natural enemies; and (iii) time is limited to locate a mate.

7.3 Methods

7.3.1 The model

Using computer simulations, we model the effects of ecological parameters on the stable aggregation strategy for larval aggregation behaviour. Parameters included are (i) the probability of mate encounter off-site; (ii) time
available for finding a mate; and (iii) population density of parasitoids and their rate of larval parasitism. Each individual is defined by a strategy characterized by two traits: (1) the preference for aggregation (A) expressed in the larval stage, and (2) the preference for on-site (localised) mating (M) expressed in the adult stage. Specific trait values for the aggregation trait (A) are denoted by \( \alpha \), and for the on-site mating trait (M) are denoted by \( \mu \). Individuals are represented as 6-bit strings of information divided into 3-bit segments, which allows eight trait values (ranging from 0 to 7) for each trait (A and M). Low values for \( \alpha \) and \( \mu \) correspond to a low preference of aggregating and on-site mating, respectively. These two traits affect two decisions, expressed as probabilities, throughout the life of a codling moth. The aggregation trait A affects the probability of accepting a pupation site (\( p_{\text{accept}} \)). The on-site mating trait M affects the probability of mating (\( p_{\text{mate}} \)). The specific functions relating the trait values to probabilities are presented in Appendix A1. The simulations occur in a spatially-explicit world which is modelled after our field surveys (Jumean et al. 2009b; pers. obs.). Various numbers of pupation sites of varying quality are randomly assigned to the world, which is described by a wrap-around grid (15 \( \times \) 9) representing the lower metre of a tree trunk (100 cm height \( \times \) 60 cm circumference), similar to the average trunk size examined by Jumean et al. (2009b). We assume that each ‘tree trunk’ comprises 15 sites with the potential to house up to one larva, 25 sites which can house up to five larvae, and 5 sites that can house up to ten larvae. At the start of a simulation, 250 individual larvae are randomly assigned trait values for both A and M, to avoid any effect of initial condition dependence. Each fifth-
instar larva is randomly placed at the top or bottom edge of a tree trunk, representing larvae arriving from the canopy and ground, respectively. Simulations were run over 500 generations, with each generation entailing larval and adult processes.

The larval processes entail searching for and finding a pupation site. Larvae move in discrete time-steps in two different phases. During phase I, each larva moves randomly at a rate of one cell per step. If a larva encounters a vacant pupation site, it can choose to accept it based on $p_{\text{accept}}$, which is a function of its $A$ trait value ($\alpha$), or it can continue to move in subsequent steps until it either finds a pupation site or reaches the end of phase I. In phase II, a larva must accept the next vacant pupation site it encounters, regardless of its $A$ trait value. This reduces selection bias against $A$ as otherwise selective individuals with a high $\alpha$ will have a high probability of perishing in their search for limited, multi-larvae pupation sites. A larva dies if it has not found a pupation site at the end of phase II.

After phase II, a larva enters and remains in the pupal stage for ~1/4 of its life, dependent on the global parameters assigned, during which mortality due to parasitism is applied based on $p_{\text{parasitism}}$. If the pupa survives the pupal stage, it ecloses as an adult.

Eclosing adult moths mate and produce next-generation offspring. They can stay on-site immediately after eclosion, or otherwise leave the site. If an adult stays, it can mate (if conspecifics are present) based on $p_{\text{mate}}$, which is a function
of both its $M$ trait value ($\mu$) and that of its potential mating partner. Whether off-site mating occurs, is determined by the probability of finding a mate off-site ($\delta$).

If moths mate, they transfer strategies to their offspring that are either identical to their own strategy or altered based on ‘crossover’ and ‘point mutations’ along the strategy strings. For example, binary string strategy 1 ‘000000’ and strategy 2 ‘111111’, could crossover resulting in the new strategies ‘001111’ and ‘111000’. Furthermore, point mutations can occur at any bit in the string, such that ‘000000’ can become ‘000100’. The probabilities used for crossover and point mutation ($P_{\text{crossover}}$ and $P_{\text{mutation}}$ respectively, Table I) do not reflect biological rates but instead are values that will lead to model convergence in a reasonable computational timeframe (Goldberg 1989). ‘Crossover’ and ‘point mutations’ are included to generate additional variation upon which selection can act, and to allow for efficient exploration of the solution space for successful strategies.

After moths have mated, the number of their offspring is rescaled to a constant population size ($N = 250$) based on the parents’ fitness. Fitness of an individual is inversely correlated to the time elapsed before locating a mate and is expressed according to our fitness function $w(\tau)$. We based this function on the finding that delayed mating in *C. pomonella* decreases the net reproductive rate of an individual (Vickers 1997). After each life stage, background mortality is applied at an average probability of 0.10 for each individual.
In all simulations, convergence is determined when mean trait values no longer change. Most simulations converged towards an asymptote within 100 generations, while a few converged within 300 generations.

7.3.2 Model simulations

We explore effects of the probability of off-site mating ($\phi$), the time available to locate a mate ($T$), and parasitoid attack parameters including number of parasitoids ($\phi$) and parasitoid attack shape parameter ($\sigma$) on the evolution of larval aggregation behaviour of adult moths. To do this, all parameter values except those of interest were kept constant for a simulation. For each simulation, we performed 20 replicates of each run for 500 generations. The stable trait value for $\alpha$ was determined by averaging over all individuals in the last 100 generations of each replicate. For each stable trait value, a 95% confidence interval was calculated. Stable trait values were plotted against the respective parameter of interest to determine its effect on the preference to aggregate ($A$).

Effect of probability of off-site mate encounter ($\delta$) on $A$

The probability of off-site mate encounter ($\delta$) was included as a parameter in the model to assess how this may affect a larva’s decision to remain in a cell and mate locally. To test the effect of $\delta$ on $A$, 20 replicates each of varying $\delta$ values (Table 7.1) were run while $T$ and $\phi$ were fixed at 25 and 5, respectively.
To test the effect of $M$ on $A$, $\mu$ was fixed at low, intermediate, or high values (i.e., 1, 3 or 6), or was left unfixed and allowed to be acted upon by selection.

**Effect of time available to locate a mate ($\xi$) on $A$**

Time available to locate a mate ($T$) was included as a parameter in the model to assess the consequences of delayed mating on a larva’s decision to aggregate or not. To test the effect of $T$ on $A$, 20 replicates each of varying $T$ values (Table 7.1) were run while $\delta$ and $\phi$ were fixed at 0.50 and 5, respectively. To test the effect of $M$ on $A$, $\mu$ was fixed at low, intermediate, or high values (i.e., 1, 3 or 6), or was left unfixed and allowed to be acted upon by selection.

**Effect of parasitoid number ($\phi$) and parasitoid attack shape parameter ($\sigma$) on $A$**

Number of parasitoids ($\phi$) was included as a parameter in the model to assess how parasitoid pressure affected larval aggregation. To test the effect of $\phi$ on $A$, 20 replicates each of varying $\phi$ values (Table 7.1) were run while $\delta$ and $T$ were fixed at 0.50 and 5, respectively. We set the baseline probability of a parasitoid finding an aggregation ($\beta$) to 0.66, estimated from Bezemer and Mills (2001). Moreover, we included a parasitoid attack shape parameter ($\sigma$) to assess the effects of various attack probability curves on larval aggregation. When $\sigma > 1$ and $\sigma < 1$, the attack shape curve is positively accelerating and decelerating, respectively, as aggregations increase in size. To test the effect of $\sigma$ on $A$, 20
replicates of the above specified parameters were run each for varying $\sigma$ values (Table 7.1). To test the effect of $M$ on $A$, $\mu$ was fixed at low, intermediate, or high values (i.e., 1, 3 or 6), or was left unfixed and allowed to be acted upon by selection.

### 7.4 Results

The average aggregation trait value ($\alpha$) at the start of each simulation was 3.5. The trait value for $\alpha$ stabilizes as high as $\alpha \approx 6$ for all treatments. When the propensity for on-site mating trait value ($\mu$) is 1 or 3, or allowed to be acted upon through selection, $\alpha$ rapidly decreases for increasing values of $\delta$ (the probability of off-site mating). When $\mu = 6$, $\alpha$ is more strongly selected for (Fig. 7.1). These results suggest that aggregation behaviour is advantageous under adverse mate finding conditions off-site. However, if an adult moth is likely to find a mate off-site (i.e., high $\phi$), selection of $\alpha$ is less pronounced and the stable $\alpha$ trait value for the population takes on a lower mean (Fig. 7.1). Similarly, as time available to find a mate ($T$) increases, the data show a decreasing trend implying weaker selection of $\alpha$ (Fig. 7.2). Irrespective of $\mu$, as a moth is allocated more time to find a mate there is weaker selection for aggregation (i.e., lower $\alpha$). As $\phi$ increases, $\alpha$ is selected for in a roughly linear fashion (Fig. 7.3). In our simple model with only one parasitoid strategy, these results suggest that individuals within aggregations accrue benefits as the probability of parasitism increases. However, this response is dependent on the parasitoid attack shape function ($\sigma$). When $\sigma > 1$ ($\sigma = 1$ corresponds to a linear relationship between the number of larvae in an
aggregation and the rate of attack by parasitoids), large aggregations are rendered more apparent to parasitoids than small aggregations or single larvae, and thus the aggregation trait value \( \alpha \) is selected less strongly as \( \phi \) increases (Fig. 7.4). Conversely, when \( \sigma < 1 \), larger \( \alpha \) values are selected as \( \phi \) increases (Fig. 7.4).

### 7.5 Discussion

Our evolutionary simulation offers one approach to investigate the effect of ecological parameters including the probability of mate encounter off-site, time available for finding a mate, and population of parasitoids and their rate of larval parasitism on the evolution of aggregation behaviour of *C. pomonella* larvae. Our results shed light on both intra- and interspecific processes that may contribute to the evolution of larval aggregation behaviour, and possibly non-random mate location, through natural selection.

Results obtained by varying the “off-site mating” parameter \( \phi \) make sense. The stable aggregation trait value should increase when the probability of off-site mate encounter is low. However, as the probability for off-site mate encounter increases, there is less selection pressure for \( \alpha \) to evolve within the population and it decreases as \( \delta \) increases. On the other hand, when the propensity for on-site mating is high (i.e., \( \mu = 3 \) or 6), or when \( \mu \) can evolve with \( \alpha \), then there is a distinct advantage for an individual to retain a high \( \alpha \). Individuals with high \( \mu \) values remain on-site longer and perform best as part of an aggregation.
The results obtained for the time-to-find-a-mate parameter (\(T\)) seem logical. If there is limited time to find a mate, then the probability for moths to encounter a mate would be greater on-site than off-site. However, if there is unlimited time to find a mate, then it is less important for a larva to cocoon in an aggregation and thus to encounter a mate on-site at the time of eclosion. When \(\mu\) is held high (i.e., \(\mu = 6\)), \(\alpha\) is selected at a slower rate because adult moths eclosing in an aggregation prefer to mate with individuals in that aggregation, thus propagating individuals with high \(\alpha\) and \(\mu\) values.

Aggregations of \(C.\) pomonella larvae are mediated by pheromone disseminating from freshly spun cocoons (Duthie et al. 2003; Jumean et al. 2004). This behaviour represents a trade-off between potential benefits (e.g., rapid mating of eclosing adults, protection from natural enemies, more suitable microclimate for pupal development) and costs (e.g., increased likelihood of detection by natural enemies). Delayed mating, related to \(T\) in our model, is disadvantageous to \(C.\) pomonella (Vickers 1997) suggesting that selection should favour means for expedient mate location. Males of \(C.\) pomonella eclosing near female pupae are arrested by sex pheromone emanating from these pupae (Duthie et al. 2003). This arrestment behaviour may expedite mating in a stochastic environment and may have contributed to the evolution of aggregation behaviour by \(C.\) pomonella larvae.

\(Cydia\) pomonella exhibits life-history traits that would favour the evolution of larval aggregation behaviour and thus rapid, on-site mating of eclosing adults. It exhibits a scramble competition polygynous mating system that does not allow
males to defend a resource of females. In this context, a male’s search for mates should be non-random and instead focus on sites with the greatest number of receptive females. These areas of potential mate encounter are typical at food resources or emergence sites (Alcock et al. 1976). Adult *C. pomonella* that eclose from larval aggregations (Duthie et al. 2003; Jumean et al. 2004, 2005a) are temporally and spatially proximate to potential mates. On-site mating should evolve if females (*i*) accept males shortly after eclosion, (*ii*) mate once or just a few times, and (*iii*) tend to occur in small groups (Thornhill and Alcock 1983).

Female *C. pomonella* exhibit all of these traits, supporting the theory that aggregation behaviour of larvae can confer fitness benefits to adults eclosing from aggregations.

Results obtained addressing the effect on parasitoid pressure, as a function of number of parasitoids (*ϕ*), support previous findings that a *C. pomonella* larva is less likely to be parasitized by the parasitoid *Mastrus ridibundus* if it cocoons in large aggregations instead of a small aggregation or in solitude (Jumean et al. 2009a). The parasitoid attack shape parameter (*α*) also affects potential benefits of larval aggregation, and attempts to quantify the apparency cost as the aggregation increases in size. Because *M. ridibundus* locates hosts via aggregation pheromone produced by *C. pomonella*, and because the pheromone within an aggregation diminishes over time, a decelerating attack shape curve for *M. ridibundus* is most realistic indicating that there may be apparency costs to aggregating. If an aggregation of *n* larvae is >*n* times more likely to be detected than a singleton, then the dilution of larvae within
an aggregation will not be sufficient to counter this apparency disadvantage, resulting in aggregation behaviour to be selected against. However, when we applied a decelerating attack shape parameter curve in our simulations, aggregation was selected for as parasitoid pressure increased. This suggests that it might be advantageous for larvae to be part of an aggregation.

The three assumptions for parasitoid avoidance and dilution, termed attack-abatement sensu Turner and Pitcher (1986), apply to the C. pomonella – M. ridibundus system and may, in part, explain associated benefits of larval aggregation. The first assumption is that the probability of a group being detected is not proportional to group size. Aggregations of C. pomonella larvae form gradually over 2-3 days (Jumean, pers. obs.). Aggregation pheromone disseminating from freshly spun silk serves as a kairomone for M. ridibundus (Jumean et al. 2005a) but completed or old cocoons likely disseminate little pheromone. Thus, pheromone concentration and attractiveness would not be proportional to group size. The second assumption is that a parasitoid’s probability of successful attack is unaffected by group size of the host. Cydia pomonella larvae and prepupae cocooned singly or as a group are equally defenceless against M. ridibundus attacks. The third assumption is that the parasitoid parasitizes only a single host per attack. This assumption applies to the codling moth – M. ridibundus system as M. ridibundus parasitizes on average 1.5 hosts per attack (Jumean et al. 2009a).

Recent work by Low (2008) further implies evolutionary benefits of grouping behaviour to a prey/host species such as C. pomonella, suggesting that
the costs of increased detectability associated with aggregations are offset by mechanisms such as dilution effects. The net survival benefits associated with grouping may contribute, in part, to selection for the formation of aggregations.

7.6 Conclusions

Taking a theoretical approach, we show that *C. pomonella* exhibits life-history traits that can favour the evolution of larval aggregation behaviour. A novel feature of this study included an examination of cross stage (i.e. larva and adult) interactions on the evolution of larval aggregation. Aggregation behaviour is selected for when the probability of off-site mate encounter is low and the time to locate a mate is short. In the scramble competition mating system of *C. pomonella*, polygynous males can be expected to efficiently locate a reliable resource of females. Moreover, larvae that spin cocoons in aggregations rather than in solitude experience a lower rate of parasitism by *M. ridibundus*, through attack-abatement advantages (Turner and Pitcher 1986; Jumean *et al.* 2009b).

Future work should aim to (i) determine the biological consequence of these results by empirically testing the model’s assumptions, (ii) investigate the effects of incorporating larva-pheromone interactions to the model, and (iii) continue to gather data on the costs and benefits of larval aggregation.
7.7 Appendix

Model functions (see Table A1)

Probability of a larva accepting site, $p_{\text{accept}}(\alpha,n)$

When a larva encounters a pupation site, it accepts the site based on some probability, $p_{\text{accept}}(\alpha,n)$, which is a function of $\alpha$ and the number of other individuals present ($n$), such that

$$p_{\text{accept}}(\alpha,n) = \begin{cases} 
1 - \frac{\alpha}{7} & \text{if } n = 0 \\
\frac{\alpha}{7} & \text{if } 0 < n \leq n_{\text{max}}
\end{cases}$$

equation A1

We assume an aggregation can contain a maximum of 10 larvae ($n_{\text{max}} = 10$). We assume that foraging larvae do not discern between the number of larvae within an aggregation based on the amount of aggregation pheromone they produce.

Probability of a cocooned larva being parasitized, $p_{\text{parasitism}}(n)$

During the pupal stage individuals risk being parasitized. This risk is expressed as a probability of being parasitized, $p_{\text{parasitism}}(n)$, during each
timestep, and is the product of: (i) the probability of a parasitoid being present in the current cell, $p_{\text{present}}$; (ii) the probability the parasitoid finds the pupa, $p_{\text{find}}(n)$; and (iii) the probability the parasitoid attacks the pupa successfully, $p_{\text{attack}}(n)$.

$$p_{\text{parasitism}}(n) = p_{\text{present}} \times p_{\text{find}}(n) \times p_{\text{attack}}(n)$$

equation A2

The probability that a particular cell contains a parasitoid is described by the total number of parasitoids in the environment ($\phi$) divided by the total number of cells in the environment ($E$). To gain a generalised understanding of the effects of a natural enemy (*Mastrus ridibundus*) on *C. pomonella* larval aggregations, we maintain simple parasitoid-host interactions and assume that parasitoids randomly and independently of one another search the environment for pupal aggregations.

$$p_{\text{present}} = \frac{\phi}{E}$$

equation A2.1

If a parasitoid is present in the current cell, its probability of finding an aggregation is described by the baseline probability of a parasitoid finding an aggregation ($\beta$) which was fixed at 0.66 and the number if other individuals present in an aggregation ($n$):

$$p_{\text{find}}(n) = \left[ \beta \left( \frac{n}{n_{\text{max}}} \right)^\sigma \right]$$
The shape of the curve depends on $\sigma$, such that $\sigma < 1$ yields a decelerating curve, $\sigma = 1$ yields a straight line, and $\sigma > 1$ yields an accelerating curve.

Once an aggregation is found by a parasitoid, we describe the probability of an individual pupa being attacked as inversely related to the number of moth pupae present ($n$), such that

$$p_{\text{attack}}(n) = \frac{1}{n}, \text{ if } n > 0$$

**Probability of mating, $p(\text{mate})$**

For a newly eclosed on-site adult moth, its probability of accepting a mate depends on its on-site mating preference ($M$), and is a linearly increasing function of this trait value ($\mu$) such that:

$$p_{\text{mate(on-site)}} = \frac{\mu}{7}$$
Once the moth moves off-site, the probability of accepting a mate is a fixed parameter of the environment such that:

\[ p_{\text{mate(on-site)}} = \delta \]

\textit{equation A3.2}

Therefore, the probability of a successful mating encounter between two moths is:

\[ p_{\text{mate}} = p_{\text{mate\{individual 1\}}} \times p_{\text{mate\{individual 2\}}} \]

\textit{equation A3.3}

\textit{Fitness function, w(\tau)}

One objective of our model is to determine whether the aggregation trait can be evolutionarily selected for based on the benefits of expedient mating. Delayed mating linearly decreases the net reproductive rate of \textit{C. pomonella} adults (Vickers et al. 1997). Therefore, our fitness function for a mated individual is based directly on the time remaining to locate a mate (\( \tau \)) and is described by the following function:

\[ w(\tau) = \frac{\tau}{T} \]

\textit{equation A4}
where $T$ is the maximum number of time steps available during the mating phase of the simulation.
Table A1. Descriptions of variables and functions used in the model.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha )</td>
<td>Aggregation trait value</td>
</tr>
<tr>
<td>( \mu )</td>
<td>On-site mating trait value</td>
</tr>
<tr>
<td>( \tau )</td>
<td>Time remaining to find a mate</td>
</tr>
<tr>
<td>( n )</td>
<td>Number of individual present in an aggregation</td>
</tr>
<tr>
<td>( P_{\text{attack}} )</td>
<td>Probability of parasitoid attacking an individual in an aggregation</td>
</tr>
<tr>
<td>( P_{\text{find}} )</td>
<td>Probability of a parasitoid finding an aggregation</td>
</tr>
<tr>
<td>( P_{\text{mate}} )</td>
<td>Probability of an individual finding a mate</td>
</tr>
<tr>
<td>( P_{\text{parasitism}} )</td>
<td>Probability of an individual being parasitized</td>
</tr>
<tr>
<td>( P_{\text{present}} )</td>
<td>Probability of a parasitoid being present in the current aggregation site</td>
</tr>
<tr>
<td>( w )</td>
<td>Fitness</td>
</tr>
</tbody>
</table>
7.8 Acknowledgements

Financial support was provided by a Natural Sciences and Engineering Research Council of Canada (NSERC) – Canada Graduate Scholarship to Z.J. and by an NSERC – Industrial Research Chair to G.G. with Contech Enterprises Inc., S.C. Johnson Canada, and Global Forest Science as industrial sponsors.

7.9 Literature cited


Table 7.1  Descriptions of parameters used in the model and their values.
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Value(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta$</td>
<td>Baseline probability of a parasitoid finding an aggregation</td>
<td>0.66</td>
</tr>
<tr>
<td>$\delta$</td>
<td>Probability of individual mating off-site</td>
<td>0.02 – 1.00</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>Parasitoid attack shape curve</td>
<td>0.25 – 1.75</td>
</tr>
<tr>
<td>$\phi$</td>
<td>Number of parasitoids</td>
<td>1 – 40</td>
</tr>
<tr>
<td>$G$</td>
<td>Maximum number of generations each simulation is allowed to run</td>
<td>500</td>
</tr>
<tr>
<td>$N$</td>
<td>Maximum number of individuals in the population at the start of each generation</td>
<td>250</td>
</tr>
<tr>
<td>$n_{max}$</td>
<td>Maximum number of individuals per aggregation site</td>
<td>10</td>
</tr>
<tr>
<td>$P_{crossover}$</td>
<td>Probability of binary string crossover between two individual strings</td>
<td>0.10</td>
</tr>
<tr>
<td>$P_{mutation}$</td>
<td>Probability of point mutation in a binary string</td>
<td>0.02</td>
</tr>
<tr>
<td>$T$</td>
<td>Time available to find a mate</td>
<td>1 – 40</td>
</tr>
</tbody>
</table>
Figure 7.1  Effect of varying values of the mating probability off-site ($\delta$) and the on-site mating preference trait value ($\mu$) on selection of the aggregation trait value ($\alpha$) (mean ± 95% CI). For “$\mu$ floats”, $\mu$ evolves with $\alpha$, whereas $\mu$ is held constant for all other runs. Data points whose CIs do not overlap are significantly different from one another. Scale of vertical axis magnified for clarity.
Figure 7.2  Effect of varying values of time available to locate a mate ($T$) and the on-site mating preference trait value ($\mu$) on selection of the aggregation trait value ($\alpha$) (mean ± 95% CI). For “$\mu$ floats”, $\mu$ evolves with $\alpha$, whereas $\mu$ is held constant for all other runs. Data points whose CIs do not overlap are significantly different from one another. Scale of vertical axis magnified for clarity.
Figure 7.3 Effect of the number of parasitoids ($\phi$) and the on-site mating preference trait value ($\mu$) on selection of the aggregation trait value $\alpha$ (mean ± 95% CI). For “$\mu$ floats”, $\mu$ evolves with $\alpha$, whereas $\mu$ is held constant for all other runs. Data points whose CIs do not overlap are significantly different from one another. Scale of vertical axis magnified for clarity.
**Figure 7.4** Effect of the number of parasitoids ($\phi$) and the shape parameter for parasitoid attack ($\sigma$) on selection of the aggregation trait value $\alpha$ (mean ± 95% CI). Data points whose CIs do not overlap are significantly different from one another. Scale of vertical axis magnified for clarity.
8: PHEROMONE-BASED TRAPPING OF LARVAL CODLING MOTH, *Cydia pomonella*, IN APPLE ORCHARDS

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8.1 Introduction

In apple orchards throughout the world, larvae of the codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae), are the primary cause of insect damage to fruit (Landolt et al., 1999). Tactics available as part of integrated pest management (IPM) programs for *C. pomonella* include pheromone-based mating disruption (Judd et al., 1997) or attract-and-kill of adult moths (Charmillot & Hofer, 1997), sterile insect technology (Judd & Gardiner, 2005), biological or conventional insecticide targeting neonate larvae (Knight et al., 1994; Lacey et al., 2004), release of parasitic wasps, like *Ascogaster quadridentata* Wesmael (Clausen, 1978) and *Mastrus ridibundus* Gravenhorst (Unruh, 1997) which parasitize egg and cocoon stages, respectively, and use of cultural controls like tree bands placed on the lower bole to trap fifth-instar larvae seeking pupation sites (Judd et al., 1997; Judd & Gardiner, 2005).

Cocoon-spinning *C. pomonella* larvae release an aggregation pheromone that attracts both the larval parasitoid *M. ridibundus* (Jumean et al., 2005b) and conspecific larvae (Duthie et al., 2003; Jumean et al., 2004, 2005a). This pheromone is comprised of eight components: 3-carene, octanal, nonanal, decanal, (E)-2-octenal, (E)-2-nonenal, sulcatone, and geranylacetone (Jumean et al., 2005b). We tested whether a synthetic blend of the larval aggregation pheromone could be used to increase the efficiency of cardboard bands for trapping *C. pomonella* larvae in apple orchards.
8.2 Materials and methods

8.2.1 Preparation of pheromone-treated cardboard bands

A polyurethane matrix was prepared by mixing a 1,3-polybutadiene holopolymer with diphenylmethane diisocyanate (proprietary materials). The matrix was made flexible by inclusion of the plasticizer acetyl tri-n-butyl citrate (Uniplex Chemical Corporation, Greensboro, NC, USA), and was stabilized by Ethanol 702 (Ethyl Corporation, Baton Rouge, LA, USA). Under continuous stirring of the mixture, the synthetic blend of *C. pomonella* larval aggregation pheromone was added at doses of 0, 10,000, 100,000, or 1,000,000 larval hour equivalents (LHE) per 30 cm of band. Ten LHE represented 22.8 ng of pheromone [sulcatone (0.81 ng), octanal (0.94 ng), 3-carene (0.95 ng), (E)-2-octenal (4.10 ng), nonanal (4.10 ng), (E)-2-nonenal (10 ng), decanal (1.40 ng), geranylacetone (0.50 ng)], equivalent to that produced by 10 cocoon-spinning larvae in 1 h except that (E)-2-octenal and (E)-2-nonenal were enhanced ×10 (Jumean et al., 2005a). All pheromone components were purchased from Aldrich (Milwaukee, WI, USA) or Bedoukian (Danbury, CT, USA), and were >95% chemically pure. A custom-built machine (30×10×10 cm) moved cardboard bands at a rate of 4.5 m min⁻¹ while applying a thin stream of the polyurethane/pheromone mixture at a rate of 9 g min⁻¹ to the band’s centre. Treated bands were covered with wax paper, cured overnight at room temperature, and then rolled and stored in Mylar® bags (West Coast Food Pak, Vancouver, BC, USA) at −20 °C.
8.2.2 Experimental sites

Experiments were conducted in two 2.5-ha commercial apple orchards [Orchard 1 (49° 55.988′N, 119° 21.636′W), Orchard 2 (49° 53.649′N, 119° 20.891′W)] in Kelowna, British Columbia, Canada. These orchards were 30–45 years old and planted at a density of 200–240 trees ha$^{-1}$, with tree × row spacings of 3 × 5 m (Orchard 1) and 5 × 5 m (Orchard 2), respectively. Apple varieties in Orchard 1 were Spartan, MacIntosh, Gala, Delicious, Granny Smith, and Golden Delicious, and in Orchard 2 MacIntosh and Delicious. Orchard 2 was certified organic, managed under guidelines of the Certified Organic Association of British Columbia (http://www.certifiedorganic.bc.ca/), but no insecticides were sprayed in either orchard during the study.

8.2.3 Experimental set-up

For every tree in each orchard, either the trunk or a main scaffold limb that was at least 30 cm in circumference was wrapped with a single corrugated cardboard band (10 cm wide × 30–46 cm, Shippers Supply, Richmond, BC, Canada) treated either with a strip of pheromone-impregnated polyurethane (treatment) or untreated polyurethane (control). A main scaffold limb was banded when the trunk circumference was >60 cm (<10%), because such large trunks would have required longer bands with increased pheromone release. All bands were attached at a height of 50–150 cm above ground. Treatment and control bands were systematically assigned to alternate trees in each row, leaving perimeter rows untreated to avoid possible edge effects. In each orchard, the
total area of treated trees was ca. 1 ha. Experiments were initiated between 23 and 30 August 2004, and terminated between 9 and 11 October 2004, at which time bands were removed and numbers of cocooning second-generation larvae destined for overwintering diapause were recorded. In Experiment 1 conducted in Orchard 1, we compared the efficiency of bands treated with 0 or 100 000 LHE of aggregation pheromone per 30 cm of band for trapping *C. pomonella* fifth-instar larvae. The relatively high pheromone dose in Experiment 1 was based on considerations that (i) cardboard bands would remain in the field for a long time; (ii) pheromone release rates would likely decline over time; and (iii) the active space over which foraging larvae are recruited would increase. In Experiment 2 conducted in Orchard 2, we tested the effect of larval pheromone dose (0, 10 000, 100 000, or 1 000 000 LHE per 30 cm of band) on capture efficiency of bands.

### 8.2.4 Measurement of pheromone release rates

Three corrugated cardboard bands (each 10 x 120 cm) were loaded with equivalent amounts of polyurethane-containing pheromone at 1 000 000 LHE per 30 cm (see above), cured overnight, rolled, and then placed in three separate glass chambers (15.5 in diameter x 20 cm high) with corrugations running parallel to air flow. Air was drawn at 24 °C through the chambers at ca. 1.5 l min⁻¹ and then through a glass column (14 x1 cm in diameter) containing Porapak Q (50–80 mesh, Waters, Milford, MA, USA). Porapak Q traps were replaced every day for 8 days, and after days 11, 14, 17, and 27. Volatiles were
eluted from each Porapak Q trap with a pentane rinse (3 ml). With tetradecane as an internal standard, pheromone components in each sample were quantified (mg per 30 cm day\(^{-1}\)) by gas chromatography, employing a Hewlett Packard (HP) 5890 Series II gas chromatograph equipped with a GC column (30 m × 0.32 mm in diameter) coated with DB-5 (J and W Scientific, Folsom, CA, USA) [temperature program: 50 °C (1 min), 10 °C min\(^{-1}\) to 200 °C (held for 5 min)]. Release rates of all pheromone components were quantified.

### 8.2.5 Statistical analyses

In Experiment 1, mean numbers of *C. pomonella* larvae cocooning in treatment (n = 91) and control bands (n = 97) were compared with a two-sample Student's t-test. Experiment 2 was analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison procedure to compare means of 0 (n = 44), 10 000 (n = 50), 100 000 (n = 46), and 1 000 000 (n = 51) LHE treatments. The experimental error rate in both experiments was set at \(\alpha = 0.05\) (Zar, 1999).

### 8.3 Results and discussion

In Experiment 1, bands treated with pheromone at 100 000 LHE were more effective in capturing *C. pomonella* larvae than untreated bands (t = 2.37, d.f. = 186, P = 0.018) (Figure 8.1). In Experiment 2, bands receiving the 1 000 000 LHE treatment captured more than twice as many larvae as untreated control bands (F = 4.53, d.f. = 3,187, P = 0.0043) (Figure 8.1). Bands receiving the 1 000
000 or 100 000 LHE treatments both captured more larvae than bands with the 10 000 LHE treatment, which did not capture significantly more larvae than control bands. Our data provide proof-of-concept that synthetic larval aggregation pheromone can be used to enhance captures of fifth-instar *C. pomonella* larvae in trapping devices. This is the first such demonstration for a larval aggregation pheromone.

Polyurethane appears suboptimal as a pheromone dispensing matrix and should be replaced with a more suitable dispenser. 3-Carene as one of the critical pheromone components (Jumean et al., 2005a) was detectable in the effluvium of cardboard bands for fewer than 3 days after initiation of release rate studies, and most other compounds were released at very high rates during the first 3 days, with rapidly declining release rates thereafter (Figure 8.2).

Larvae exited apples about 1 week after bands were placed on trees (Gavin Young, Field Manager, Okanagan-Kootenay Sterile Insect Release Program, pers. comm.), suggesting that they responded to pheromone release rates that had already declined to biologically more ‘appropriate’ levels. Although pheromone release rates after day 4 (Figure 8.2) were still up to 1000 times greater than the dose (228 ng day\(^{-1}\)) that attracted larvae in laboratory bioassays (Jumean et al., 2005a), these release rates were apparently effective in covering the greater active space over which ‘field lures’ would be expected to attract foraging larvae. To ensure continuous optimal efficiency of the larval trapping devices, dispensers with constant pheromone release rates ought to be developed.
Use of tree bands to capture and reduce populations of overwintering larvae is an effective supplemental tactic within IPM programs for *C. pomonella* (Judd et al., 1997; Judd & Gardiner, 2005); our data suggest that synthetic larval pheromone can be used to enhance the efficiency of this control tactic.

### 8.4 Acknowledgements

Thanks to Gavin Young for assistance in locating field sites, Dennis and Kathleen Behnke and Surjit Nagra for allowing access to their orchards, and John Borden for reviewing the manuscript. Financial support was provided by a BP Beirne Prize in Pest Management to ZJ, a Natural Sciences and Engineering Research Council of Canada – Industrial Research Chair to G. Gries, with Phero Tech Int., SC Johnson Canada, and Global Forest Science (6F-18-2004-216; 6F-18-2004-217) as industrial sponsors, and by a grant from the Washington Tree Fruit Research Commission to GJR. Judd and G. Gries.

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USDA, Washington, DC, USA.


Figure 8.1  Mean number (± SE) of *Cydia pomonella* larvae captured in corrugated cardboard bands treated either with an untreated strip of polyurethane (control) or polyurethane containing 100 000 larval hour equivalent (LHE) of synthetic larval aggregation pheromone (Experiment 1) or various doses of pheromone (Experiment 2). Bars with different letters in each experiment represent treatments that were significantly different by Student's t-test (Experiment 1), and by ANOVA followed by Tukey's multiple comparison procedure (Experiment 2). Number of replicates (n) for each treatment is shown within bars. One LHE = one larval hour equivalent = pheromone equivalent to that produced by one cocoon-spinning larva in 1 h.
**Figure 8.2** Release rates of pheromone components (mean ± SE) from a pheromone-impregnated polyurethane strip on cardboard bands (n = 3). Porapak Q traps were replaced every day for 8 days, and then after days 11, 14, 17, and 27. Bands were impregnated with 1 000 000 LHE (see text) of pheromone.
9: COCOON SPINNING LARVAE OF ORIENTAL FRUIT MOTH AND INDIANMEAL MOTH DO NOT PRODUCE AGGREGATION PHEROMONE¹

9.1 Abstract

1. Mature larvae of the Oriental fruit moth (OFM) *Grapholita molesta* (Lepidoptera: Tortricidae) and the Indianmeal moth (IMM) *Plodia interpunctella* (Lepidoptera: Pyralidae) leave their food source in search of suitable pupation sites in which to spin cocoons. These sites are typically well-concealed cracks and crevices within the environment. Such cocooning behaviour is also observed in larvae of the codling moth (CM) *Cydia pomonella* (Lepidoptera: Tortricidae) which aggregate prior to pupation in response to a pheromone blend produced by cocoon-spinning conspecific larvae.

2. In laboratory experiments, we tested whether cocoon-spinning OFM and IMM larvae produce aggregation pheromones and whether CM larvae are cross-attracted to closely related OFM larvae.

3. Fifth-instar OFM and IMM larvae were not attracted to, or arrested by, cocoon-spinning conspecifics. Moreover, fifth-instar CM larvae were not cross-attracted to either cocoon-spinning OFM or IMM larvae.

4. Analyses of volatiles released from cocoon-spinning OFM and IMM larvae revealed that both OFM and IMM lack components that are present in the aggregation pheromone of CM larvae. This information may help explain why CM larvae are not cross-attracted to cocooning OFM or IMM larvae.
9.2 Introduction

Pheromonal communication among larvae of holometabolous insects has been investigated in just a few species. For example, larvae of the coniferophagous great spruce bark beetle *Dendroctonus micans* produce an aggregation pheromone and feed in groups rather than in individual tunnels, resulting in an increased growth rate (Deneubourg et al., 1990; Storer et al., 1997). Similarly, phloem-feeding larvae of the greater peachtree borer moth *Synanthedon exitiosa* produce a 2-component pheromone [(Z)-9-octadecenyl acetate, (Z,Z)-9,12-octadecadienyl acetate] that attracts conspecific larvae in laboratory bioassays (Derksen, 2006). However, fitness benefits to individuals that use this pheromone are not known. Finally, cocoon-spinning larvae of the codling moth (CM) *Cydia pomonella* (Lepidoptera: Tortricidae) produce an 8-component aggregation pheromone that disseminates from fresh cocoon and attracts conspecific larvae seeking pupation sites (Duthie et al., 2003; Jumean et al., 2004, 2005a,b, 2007, 2008). The fitness benefits larvae accrue by responding to the pheromone are still unknown, but may include efficient location of suitable pupation sites, reduced risk of parasitism, and expedient mating of eclosing adults (Duthie et al. 2003).

In our insectary, we observed that > 30 larvae of the Indianmeal moth (IMM) *Plodia interpunctella* (Lepidoptera: Pyralidae) had crawled through a narrow 2 × 2 cm opening and cocooned side-by-side on a roll of Velcro™ tape. This behaviour was reminiscent of aggregating CM larvae, and suggested that IMM larvae may also produce an aggregation pheromone. We predicted the
same type of behaviour for larvae of the Oriental fruit moth (OFM) *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae), which is a close relative of CM and inhabits a similar ecological niche.

OFM and IMM are cosmopolitan pest species (Rothschild and Vickers, 1991; Mohandass et al., 2007). OFM larvae attack shoots and fruits of many temperate fruit trees, including peach, apricot, nectarine and apple (Rothschild and Vickers, 1991). Mature OFM larvae exit the host plant and spin cocoons in bark crevices or in the duff below host trees. Pheromonal communication among OFM larvae has not yet been demonstrated but kairomonal attraction of larvae to twig and fruit semiochemicals has previously been shown (Bouzouane et al., 1987).

IMM larvae feed mainly on stored food products but also on rice and grains in agricultural fields (Vick et al., 1987; Anderson and Löfqvist, 1996). Mature fifth-instar larvae exit their food source and, in this wandering stage, seek pupation sites in crevices within their habitat (Williams, 1964; Z.J. pers. obs.). Pre-wandering fifth-instar larvae repel each other by semiochemicals derived from droplets of mandibular gland excretions (Mossadegh, 1980), providing evidence for pheromonal communication among pre-wandering larvae. Wandering-stage larvae have the same semiochemicals at a different ratio (Howard and Baker 2004), but the response to them has not yet been investigated.

Based on the taxonomic relationship between CM and OFM (Komai, 1999) and laboratory observations of aggregated IMM pupae, we tested the hypotheses
i) that cocoon-spinning larvae of OFM and IMM produce aggregation pheromone attractive to conspecifics, and ii) that CM larvae are cross-attracted to volatiles from cocoon-spinning larvae of closely related OFM but not to those from distantly related IMM larvae.

### 9.3 Materials and methods

#### 9.3.1 Experimental insects

Specimens of OFM were collected in New Jersey, USA, and kept in laboratory culture at the University of Alberta, Edmonton, Alberta, Canada until transfer to the Global Forest Quarantine Facility at Simon Fraser University (SFU), Burnaby, British Columbia, Canada. Insects were maintained at 22°C with a photoregime of 16L:8D. Wax paper sheets bearing OFM eggs were washed with a 0.001% bleach solution, rinsed with distilled water and placed in 4-L plastic rearing vessels with mesh lids containing a lima bean-based larval diet. This diet differed from that of Shorey and Hale (1965) in that Vanderzant Vitamin Mixture for Insects (5 % by volume) (Sigma-Aldrich, Inc., St. Louis, MO, USA) was added, formaldehyde was omitted, and carrageenan – Type I (3.5 %) (Sigma-Aldrich, Inc., St. Louis, MO, USA) was substituted for agar. Three strips (7 × 5 cm diam) of coiled single-faced cardboard serving as pupation sites were placed in each container before larvae exited the diet. Cardboard rolls with pupae were then placed in a rearing cage (24 × 24 × 36 cm) with a wax paper front serving as an oviposition substrate. Emergent adult moths were provided with a 10%
sucrose solution and distilled water *ad libitum*, and were free to mate and oviposit.

Specimens of IMM were collected in Vancouver, Canada and kept in laboratory culture in SFU's insectary. Insects were reared at 27°C with a photoregime of 17L:7D. Mixed groups of 10-25 males and 10-25 females were placed into 4-L glass rearing vessels containing diet covered with mesh lids and were free to mate and oviposit. Larvae were reared on a diet consisting of whole wheat flower (27.5 % by volume), corn meal (27.5 %), Purina One™ dog food (14 %), brewers yeast (7 %) (Sigma-Aldrich, Inc., St. Louis, MO, USA), rolled oats (7 %), liquid honey (7 %), glycerol (7 %) (96% chemically pure; Caledon Laboratories Ltd, Georgetown, ON, Canada), and wheat germ (3 %). Fifth-instar larvae were transferred to Petri dishes (2.5 × 9 cm diam) containing three cardboard strips (each 3 cm²) as pupation substrate. Emergent adult moths were placed into new rearing vessels.

**9.3.2 Test stimuli and experimental design**

A bioassay stimulus for experiments 1-4 and 7-8 (Table 9.1) was generated by removing 5-7 fifth-instar OFM (exp 1, 3, 7) or IMM (exp 2, 4, 8) larvae from diet and allowing them to cocoon on a corrugated cardboard strip (2.5 cm²) for 2-3 d. Treatment and empty control strips (Table 9.1) were randomly assigned to one of two 4-mL vials in still-air two-choice olfactometers (14 cm diam) (Pierce et al., 1981; Duthie et al., 2003). The olfactometer consists of a Petri dish (14 cm diam) with two holes (each 1.5 cm diam) in the bottom spaced
6.7 cm apart. Each hole is connected to a 4-mL vial fitted with a perforated microcentrifuge tube. The presence or absence of this tube in each vial prevented or allowed physical contact between bioassay insects and test stimuli. For each replicate, one fifth-instar OFM (exp 1, 3), IMM (exp 2, 4), or CM (exp 7, 8) was placed in the centre of the olfactometer, and its pupation site was recorded after 24 h (exp 1-4, 7, 8) and 48 h (exp 1, 2) (Table 1). In experiments 5 and 6, 10 fifth-instar OFM larvae (exp 5) or IMM larvae (exp 6) were placed in the centre of each of 10 plastic Petri dishes (9 cm diam) (Table 9.1). After 4 d, cocooning larvae were recorded as solitary or in an aggregation. An aggregation was defined as ≥ 2 cocoons contacting each other. All experiments were conducted at 22-25°C in complete darkness.

Experiments 1-4 (Table 9.1) tested the hypotheses that pupation site seeking fifth-instar OFM or IMM larvae respond to airborne pheromone (exp 1, 2) or contact pheromone (exp 3, 4) emanating from, or associated with, cocoon-spinning conspecific larvae. Experiments 5 and 6 (Table 9.1) tested the hypothesis that fifth-instar OFM (exp 5) and IMM (exp 6) spin cocoons in aggregates rather than in solitude. Finally, experiments 7 and 8 tested the hypothesis that pupation site seeking fifth-instar CM larvae are cross-attracted to volatiles produced by cocoon-spinning OFM larvae (exp 7) but not those of IMM larvae (exp 8).
9.3.3 Collection and analysis of volatiles

Three-hundred OFM or IMM larvae in each of three replicates were placed in a custom made Pyrex® glass aeration chamber (15.5 × 20 cm) (Science Technical Center, Simon Fraser University, Burnaby, Canada), and charcoal-filtered air was drawn at 1.5 l/min through the chamber and a glass column (14 × 1.3 cm i.d.) containing Porapak Q (50-80 mesh; Waters Associates, Inc., Milford, MA, USA). After 72 h, volatiles were eluted from the Porapak Q trap with 3 ml of pentane. Extracts were concentrated under a nitrogen stream so that 1 µl was equivalent to ~11 cocoon-spinning larval hour equivalents (11 CSLHE = volatiles released from 11 cocoon-spinning larvae during 1 h). Extracts were analyzed by coupled gas chromatography-mass spectrometry (GC-MS) in full-scan electron impact mode using a Varian Saturn 2000 Ion Trap GC-MS fitted with a DB-5 column (30 m × 0.25 mm i.d., J&W Scientific, Folsom, CA, USA). The composition of volatile blends emitted by OFM and IMM larvae was compared with that emitted by CM larvae, as reported by Jumean et al. (2004, 2005a) and to a control aeration.

9.3.4 Statistical analyses

The number of larvae responding to stimuli in bioassay experiments 1-4, 7 and 8, and aggregation behaviour of larvae in experiments 5 and 6 were analyzed with the \( \chi^2 \) goodness-of-fit test, using Yates correction for continuity (\( \alpha = 0.05 \)) (Zar, 1999).
9.4 Results

Fifth-instar pupation site seeking OFM larvae were not attracted to, or arrested by, volatiles emanating from cocoon-spinning conspecific larvae (at 24 h: $\chi^2 = 0.25$, df = 1, $P = 0.62$; at 48 h: $\chi^2 = 2.16$, df = 1, $P = 0.14$) (Fig. 9.1, exp 1). Fifth-instar pupation site seeking IMM larvae cocooned more often in control vials than in treatment vials containing cocoon-spinning conspecific larvae (at 24 h: $\chi^2 = 9.33$, df = 1, $P < 0.01$; at 48 h: $\chi^2 = 10.24$, df = 1, $P < 0.01$) (Fig. 9.1, exp 2).

When cocooning conspecifics were accessible (exp 3, 4), OFM and IMM larvae did not cocoon more often in treatment than in control vials (exp 3: $\chi^2 = 0.12$, df = 1, $P = 0.73$; exp 4: $\chi^2 = 2.56$, df = 1, $P = 0.11$) (Fig. 9.2). Moreover, OFM and IMM larvae confined in Petri dishes (exp 5, 6) did not cocoon in aggregates with conspecifics (exp 5: $\chi^2 = 20.76$; df = 1; $P < 0.001$; exp 6: $\chi^2 = 36.41$; df = 1; $P < 0.001$) (Fig 9.3).

Fifth-instar CM larvae were not cross-attracted to cocoon-spinning OFM larvae ($\chi^2 = 0.46$; df = 1; $P = 0.50$) (exp 7) or IMM larvae ($\chi^2 = 1.17$; df = 1; $P = 0.28$) (exp 8) (Fig 9.4). Analyses of volatiles from cocoon-spinning OFM larvae revealed that two essential components [3-carene, (E)-2-octenal] of the CM larval aggregation pheromone were absent in each of the three replicates. Similarly, analyses of volatiles from cocoon-spinning IMM larvae revealed that one essential component (octanal or 3-carene) was absent in each of the three replicates (Table 9.2).
9.5 Discussion

Our data do not support the hypothesis that Oriental fruit moth (OFM) or Indianmeal moth (IMM) fifth-instar larvae are attracted to, or arrested by, cocoon-spinning conspecific larvae. Both OFM and IMM larvae were not attracted or arrested by cocoon-spinning conspecifics (Fig 9.1, exp 1, 2). Expectedly then, OFM and IMM larvae cocooned in solitude rather than in aggregates when they were confined with conspecific larvae (Fig 9.3, exp 5, 6).

The lack of pheromone-based aggregation behaviour among OFM and IMM larvae is in contrast to the production of and response to aggregation pheromone by codling moth (CM) larvae (Duthie et al., 2003; Jumean et al., 2004; 2005a), a phenomenon hypothesized to facilitate the earliest possible mating between eclosed male and female CM (Duthie et al., 2003) and thus to minimize adverse fitness consequences associated with delayed mating (Vickers, 1997; Knight, 1997). Although delayed mating has similar adverse fitness consequences for OFM and IMM as it has for CM (Fraser and Trimble, 2001; Huang and Subramanyam, 2003), it appears that the concept of an early mating strategy, which might explain CM larval aggregation, is not applicable to OFM and IMM because they do not exhibit larval aggregations.

There are several explanations for our results. Firstly, competitive pre-wandering stage IMM larvae may not be able to change strategy (Anderson & and Löfqvist, 1996) as they proceed to the cocoon-spinning stage. IMM larvae develop within, and compete for, the same resource, sometimes even
cannibalising each other (Bjørnstad et al., 1998). Throughout their larval
development, they produce spacing pheromone that helps regulate colonization
densities in the resource (Mossadegh, 1980; Howard and Baker, 2004). IMM
larvae may lack the physiological mechanisms to cease production of the spacing
pheromone, and/or to produce and respond to aggregation pheromone in their
final larval instar prior to pupation. The change in ratio of semiochemicals in
mandibular glands as pre-wandering stage larvae proceed to the wandering
stage (Howard and Baker 2004) apparently does not trigger a change in
behavioural response.

Secondly, assuming that aggregations of cocoon-spinning larvae
represent a trade-off between costs (e.g., increased risk of predation and
parasitism) and benefits (e.g. early mate acquisition), then the costs may
outweigh the benefits for IMM larvae. IMM larvae in food sources treated with
conspecific silk experience greater rates of parasitism by the parasitoid *Nemeritis
canescens* than larvae residing in silk-free food sources (Mudd and Corbet, 1973;
Mossadegh, 1980). This may also be applicable to OFM, but few parasitoids are
known to attack OFM late instar larvae or prepupae, and attraction of parasitoids
has yet to be linked to cocoon-spinning behaviour. Aggregations of CM larvae
attract the parasitoid *Mastrus ridibundus* that exploit the aggregation pheromone
as a host location kairomone (Jumean et al., 2005b), but members of this
aggregation may not necessarily be subject to a greater risk of parasitism. On the
contrary, CM larvae in large aggregations experience a lower overall rate of
parasitism than larvae in small aggregations due to inverse density-dependent
dilution effects and structural refugia created by aggregated larvae pupating side-by-side and on top of one another (Z.J., unpublished data).

Lack of cross-attraction of CM larvae to cocoon-spinning OFM or IMM larvae (Fig 9.4, exp 7, 8) suggested that OFM and IMM larvae do not produce any or all of the components of the CM larval aggregation pheromone (see Jumean et al., 2004). Although there was a lot of overlap in headspace volatiles of cocoon-spinning CM, OFM and IMM larvae (Table 9.2), one or two components [3-carene and (E)-2-octenal] of the CM larval aggregation pheromone were consistently absent in volatile blends of OFM or IMM larvae. CM larvae did not respond to such blends because 3-carene and (E)-2-octenal are essential components of the CM pheromone, as shown in experiments that determined the composition of the CM larval aggregation pheromone (Jumean et al. 2005a).

In conclusion, life-history traits and/or observations of cocooning behaviour of OFM and IMM prompted us to hypothesize that cocoon-spinning larvae of both species produce and respond to aggregation pheromone. Our experimental data, however, do not support this hypothesis. Pheromone-mediated larval aggregations in holometabolous insects remain a rare biological phenomenon.

9.6 Acknowledgements

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Figure 9.1  Response of individual fifth-instar Oriental fruit moth (OFM) (exp 1) and Indianmeal moth (IMM) (exp 2) in two-choice olfactometers (Duthie et al., 2003) to cocooning conspecifics, disallowing contact with them. Numbers of larvae responding to test stimuli are given within bars. An asterisk (*) indicates a significant response to a test stimulus; $\chi^2$ goodness-of-fit test with Yates correction for continuity, $P < 0.01$. 
Exp 1  Two-choice olfactometer; contact with test stimuli not allowed

| Response at 24 h | 5 cocooning OFM larvae | 5 | Control | 8 |
| Response at 48 h | 5 cocooning OFM larvae | 11 | Control | 20 |

Exp 2  Two-choice olfactometer; contact with test stimuli not allowed

| Response at 24 h | 5 cocooning IMM larvae | 3 | Control | 18 * |
| Response at 48 h | 5 cocooning IMM larvae | 4 | Control | 21 * |
Figure 9.2  Response of individual fifth-instar Oriental fruit moth (OFM) (exp 3) and Indianmeal moth (IMM) (exp 4) in two-choice olfactometers (Duthie et al., 2003) to cocooning conspecifics, allowing contact with them. Numbers of larvae responding to test stimuli are given within bars. There was no significant response to a test stimulus; $\chi^2$ goodness-of-fit test with Yates correction for continuity.
**Exp 3** Two-choice olfactometer experiment; contact with test stimuli allowed

- 5 cocooning OFM larvae: 16
- Control: 17

**Exp 4** Two-choice olfactometer experiment; contact with test stimuli allowed

- 5 cocooning IMM larvae: 15
- Control: 24
**Figure 9.3** Number of fifth-instar Oriental fruit moth (OFM) (exp 5) or Indianmeal moth (IMM) (exp 6) cocooning singly or in aggregates of ≥2 individuals in Petri dishes (9 cm diam.), containing 10 larvae per replicate. Total numbers of individuals cocooning singly or in aggregates are given within bars. An asterisk (*) indicates a significant preference for a specific cocooning behaviour; $\chi^2$ goodness-of-fit test with Yates correction for continuity, $P < 0.001$. 
Response of individual fifth-instar codling moth (CM) in two-choice olfactometers (Duthie et al., 2003) to cocoon-spinning larvae of Oriental fruit moth (OFM) (exp 7) or Indianmeal moth (IMM) (exp 8), disallowing contact with them. Numbers of larvae responding to test stimuli are given within bars. There was no significant response to a test stimulus; \( \chi^2 \) goodness-of-fit test with Yates correction for continuity.
Exp 7  Two-choice olfactometer; contact with test stimuli not allowed

<table>
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<tr>
<th>Test stimulus</th>
<th>5 cocooning OFM larvae</th>
<th>Control</th>
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<tbody>
<tr>
<td>Number of fifth-instar CM larvae responding</td>
<td>9</td>
<td>8</td>
</tr>
</tbody>
</table>

Exp 8  Two-choice olfactometer; contact with test stimuli not allowed

<table>
<thead>
<tr>
<th>Test stimulus</th>
<th>5 cocooning IMM larvae</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of fifth-instar CM larvae responding</td>
<td>18</td>
<td>10</td>
</tr>
</tbody>
</table>
Table 9.1 Predictions, test stimuli, bioassay insects and type of olfactometer deployed in experiments 1-8.
<table>
<thead>
<tr>
<th>Exp</th>
<th>Prediction</th>
<th>Treatment</th>
<th>Control</th>
<th>Bioassay Insects</th>
<th>n&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NR&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Olfactometer Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pupation site-seeking fifth-instar OFM larvae respond to airborne pheromone from cocoon-spinning fifth-instar OFM larvae</td>
<td>Corrugated cardboard (2.5 cm²) with 5 OFM larvae cocooning for 2-3 d</td>
<td>Corrugated cardboard (2.5 cm²)</td>
<td>One fifth-instar OFM larva per replicate</td>
<td>56</td>
<td>14</td>
<td>Pitfall two choice&lt;sup&gt;c&lt;/sup&gt;; physical contact with test stimuli prevented</td>
</tr>
<tr>
<td>2</td>
<td>Pupation site-seeking fifth-instar IMM larvae respond to airborne pheromone from cocoon-spinning fifth-instar IMM larvae</td>
<td>Corrugated cardboard (2.5 cm²) with 5 IMM larvae cocooning for 2-3 d</td>
<td>Corrugated cardboard (2.5 cm²)</td>
<td>One fifth-instar IMM larva per replicate</td>
<td>34</td>
<td>54</td>
<td>Pitfall two choice&lt;sup&gt;c&lt;/sup&gt;; physical contact with test stimuli prevented</td>
</tr>
<tr>
<td>3</td>
<td>Pupation site-seeking fifth-instar OFM larvae respond to contact cues from cocoon-spinning fifth-instar OFM larvae</td>
<td>Corrugated cardboard (2.5 cm²) with 5 OFM larvae cocooning for 2-3 d</td>
<td>Corrugated cardboard (2.5 cm²)</td>
<td>One fifth-instar OFM larva per replicate</td>
<td>33</td>
<td>4</td>
<td>Pitfall two choice&lt;sup&gt;c&lt;/sup&gt;; physical contact with test stimuli allowed</td>
</tr>
<tr>
<td></td>
<td>Pupation site-seeking fifth-instar larvae respond to contact cues from cocoon-spinning fifth-instar larvae</td>
<td>Corrugated cardboard (2.5 cm²) with 5 IMM larvae cocooning for 2-3 d</td>
<td>Corrugated cardboard (2.5 cm²)</td>
<td>39</td>
<td>41</td>
<td>Pitfall two choice; physical contact with test stimuli allowed</td>
<td></td>
</tr>
<tr>
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<td>---------------------------------------------------------------------</td>
<td>--------------------------------</td>
<td>----</td>
<td>----</td>
<td>-------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Fifth-instar OFM larvae cocoon in aggregates rather than in solitude</td>
<td>N/A</td>
<td>N/A</td>
<td>Ten fifth-instar OFM larva per replicate</td>
<td>12</td>
<td>N/A</td>
<td>Petri dish (9 cm diam)</td>
</tr>
<tr>
<td>6</td>
<td>Fifth-instar IMM larvae cocoon in aggregates rather than in solitude</td>
<td>N/A</td>
<td>N/A</td>
<td>Ten fifth-instar IMM larva per replicate</td>
<td>12</td>
<td>N/A</td>
<td>Petri dish (9 cm diam)</td>
</tr>
<tr>
<td>7</td>
<td>Pupation site-seeking fifth-instar CM larvae are cross-attracted to cocoon-spinning fifth-instar OFM larvae</td>
<td>Corrugated cardboard (2.5 cm²) with 5 OFM larvae cocooning for 2-3 d</td>
<td>Corrugated cardboard (2.5 cm²)</td>
<td>35</td>
<td>10</td>
<td>Pitfall two choice; physical contact with test stimuli prevented</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Pupation site-seeking fifth-instar CM larvae are cross-attracted to cocoon-spinning fifth-instar IMM larvae</td>
<td>Corrugated cardboard (2.5 cm²) with 5 IMM larvae cocooning for 2-3 d</td>
<td>Corrugated cardboard (2.5 cm²)</td>
<td>45</td>
<td>18</td>
<td>Pitfall two choice; physical contact with test stimuli prevented</td>
<td></td>
</tr>
</tbody>
</table>

\[^a\] n = number of replicates

\[^b\] NR = number of non-responding insects

\[^c\] Olfactometer as described by Duthie et al. (2003).
Table 9.2 Presence (+) or absence (−) of aggregation pheromone components of larval codling moth in headspace volatiles of cocoon-spinning larvae of Oriental fruit moth (OFM), Indianmeal moth (IMM) and a control aeration. Three separate samples of headspace volatiles each from OFM and IMM were analyzed by coupled gas chromatography-mass spectrometry. Minimum detectable amount in mass-spectrometry equals approximately 50 pg; trace amounts of nonanal and decanal were lower than in insect aerations.
### Aggregation pheromone components of larval codling moth

<table>
<thead>
<tr>
<th></th>
<th>Sulcatone</th>
<th>Octanal</th>
<th>3-Carene</th>
<th>(E)-2-Octenal</th>
<th>Nonanal</th>
<th>(E)-2-Nonenal</th>
<th>Decanal</th>
<th>Geranyl-acetone</th>
</tr>
</thead>
<tbody>
<tr>
<td>OFM 1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>OFM 2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>OFM 3</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IMM 1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IMM 2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>IMM 3</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Control</td>
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<td>—</td>
<td>+</td>
<td>—</td>
<td>+</td>
<td>—</td>
</tr>
</tbody>
</table>
10: CONCLUSIONS AND FUTURE WORK

10.1 Conclusions

In my thesis, I have presented an integrated analysis of the chemical composition, function, costs and benefits, and potential practical application of the aggregation pheromone of *Cydia pomonella* larvae. Larval-mediated aggregation behaviour is rare in the Insecta, making it difficult to study the factors leading to the evolution of such behaviour. With *C. pomonella* being readily reared in the laboratory, abundantly present in apple orchards, and of such economic importance, the opportunity presented itself to thoroughly study this larval communication system.

Surveying apple trees in two unmanaged orchards in Wenatchee and Yakima (Chapter 2), I have found aggregates of larvae significantly more often than solitary larvae. These data supported the conclusion (*i*) that larvae seek pupation sites not by chance but in part in response to pheromone signal and microhabitat cues, and (*ii*) that the probability of aggregates forming is likely proportional to the population density of *C. pomonella*. The data also provided impetus and incentive to carefully study this larval communication system.
Insect aggregation pheromones are produced by members of one or both sexes that induce members of both sexes to form aggregations. While it was known that pupation site-seeking *C. pomonella* larvae aggregate in response to pheromone produced by cocoon-spinning conspecific larvae (Duthie et al. 2003), it was not known whether *(i)* both male and female larvae produce and respond to the larval pheromone and *(ii)* both sexes produce the same pheromone blend. In Chapter 3, I have shown that male and female cocoon-spinning larvae produce identical volatile profiles and respond similarly to such blends, indicating that both male and female larvae produce and respond to an aggregation pheromone rather than a sex pheromone.

Identification of the cocoon-derived pheromone proved challenging because larval antennae were too small to be used effectively in GC-EAD analyses of cocoon volatiles. However, on the assumption that the late instar/prepupal parasitoid *M. ridibundus* exploits the very same odours produced by or associated with cocoon-spinning larvae as a kairomone during host-foraging, and because I had previously identified the kairomone blend (Jumean et al. 2005b), I could relatively quickly determine the essential components of the complex larval aggregation pheromone (Chapter 4). At least eight components need to be present at specific ratios to elicit an attraction response by larvae. To my knowledge, this is the first larval aggregation pheromone identified in a holometabolous insect.
Aggregation pheromones elicit behavioural responses in receivers. Arrestment responses are mediated by encounter of, and arrestment at, the pheromone source. Attractant aggregation pheromones, in contrast, attract receivers from a distance. In Chapter 5, I show that fifth-instars (i) move faster and farther upwind toward cocooning conspecifics compared to blank controls, (ii) select more often as first- and final choices of pupation sites those with cocooning conspecifics than those without, and (iii) anemotactically respond to, and prefer side arms with, cocooning conspecifics to those without. These data provide evidence that *C. pomonella* larvae are attracted to, rather than merely arrested by, larval aggregation pheromone. They also help explain the clumped distributions of larvae on tree trunks that I report in Chapter 2. Clumped distributions would likely not occur if they were based merely on chance encounter of cocoon-spinning larvae by foraging larvae.

Because foraging female *M. ridibundus* are able to eavesdrop on an aggregation pheromone produced by cocooning *C. pomonella* larvae (Jumean *et al.* 2005b), I investigated (Chapter 6) whether larvae that cocoon in aggregation experience a greater rate of parasitism than larvae that cocoon in isolation. While I found that larvae in aggregations are more readily located than solitary larvae by female *M. ridibundus* and that large aggregations are more apparent than small ones, larval cocooning in aggregation or isolation had no effect on the mean rate of parasitism and the mean number of eggs deposited per parasitized
host. The increased risk of aggregated larvae to be detected by *M. ridibundus* is offset by diluted parasitism risk and structural refugia effects that larvae in aggregations experience. As an egg-limited parasitoid, female *M. ridibundus* can parasitize on average only one larva in an aggregation, with the likelihood of parasitism for each larva being inversely proportional to the number of larvae in that aggregation.

Allee effects and non-panmictic mating are some factors predicted to favour the evolution of non-social aggregations (Wertheim et al. 2005). Modelling through genetic algorithms life history parameters of *C. pomonella* that could be conducive to the evolution of larval aggregation behaviour, I provide evidence (Chapter 7) that larval aggregation behaviour can evolve when it accrues improved protection from natural enemies and greater reproductive success.

The use of cardboard bands on trees to capture and reduce populations of overwintering larvae is an effective supplemental tactic within IPM programs for *C. pomonella* (Judd et al., 1997; Judd & Gardiner, 2005). In Chapter 8, I tested whether a synthetic blend of the larval aggregation pheromone could be used to increase the efficiency of these cardboard bands. My data provide proof-of-concept that synthetic larval aggregation pheromone can be used to enhance captures of fifth-instar *C. pomonella* larvae in trapping devices. This is the first such demonstration for a larval aggregation pheromone. However, several challenges must be addressed before synthetic larval aggregation pheromone
can be integrated in IPM programs for *C. pomonella* (see Future Work).

Pheromonal communication among larvae of holometabolous insects has been investigated in just a few species. Based on the taxonomic relationship between *C. pomonella* and *G. molestata* (Komai, 1999), and my laboratory observations of aggregated *P. interpunctella* pupae, I tested the hypotheses (*i*) that cocoon-spinning larvae of *G. molestata* and *P. interpunctella* produce aggregation pheromone attractive to conspecifics, and (*ii*) that *C. pomonella* larvae are cross-attracted to volatiles from cocoon-spinning larvae of the closely related *G. molestata*. My experimental data (Chapter 9), however, did not support either hypothesis. Pheromone-mediated larval aggregations in holometabolous insects remain a rare biological phenomenon.

### 10.2 Future work

Many new research questions have arisen from my thesis. Larval aggregation was predicted to be part of a reproductive strategy that facilitates mate encounter of adult moths. Results obtained from a theoretical model that tested effects of ecological parameters on the evolution of larval aggregations (Chapter 7) support this prediction but empirical data are still lacking. Additional potential benefits associated with larval aggregations that are worth investigating include a favourable microclimate and protection from natural enemies other than *M. ridibundus*, or the possibility that the pheromone serves as a proxy for a
suitable pupation site. The costs of larval aggregations are largely unexplored. They may include greater transmission of pathogens and greater apparency to predatory birds, or increased competition upon adult emergence. All these aspects could be investigated at a micro-scale (the aggregation patch level) or a macro-scale (the tree level).

The relationship of larvae within aggregations on a tree is still unknown. Larvae may be siblings or cousins. If so, larval aggregations would present opportunities for kin selection.

Delayed mating is costly to female *C. pomonella* in that their fecundity is reduced. Little is known whether delayed mating incurs adverse fitness consequences for males. If larval aggregations indeed expedite mate encounter of emergent adults, then one might predict (but would have to test experimentally) that individuals which have pupated in aggregations have greater fitness than their counterparts which have pupated in solitude.

The aggregation pheromone was identified from larvae that were reared on artificial diet in the Sterile Insect Release (SIR) program in Osoyoos, BC. The SIR colony was established in 1992 and since then supplemented with several introductions of feral codling moths. It was conceivable (but not very likely) that SIR larvae produce a different pheromone blend than feral larvae due to different diets or selection pressures. However, significant attraction of feral larvae in field experiments to to cardboard bands treated with SIR larvae type pheromone
(Chapter 8) implies that SIR and feral larvae likely produce very similar pheromones. Moreover, most insects biosynthesize pheromones \textit{de novo}, and do not rely on specific pheromone precursors in their diet. Nonetheless, the pheromone blend of feral larvae should be analyzed and compared with that of SIR larvae.

I have shown that cardboard bands with synthetic pheromone attracted significantly more larvae for cocooning than control bands, suggesting that pheromone-based mass trapping of larvae has potential in IPM programs for \textit{C. pomonella}. Yet, release rate studies of synthetic pheromone indicated that polyurethane was not the optimal dispenser, and that different formulations should be considered. Moreover, it might be prudent to test whether co-attractants such as fruit volatiles enhance the attractiveness of the pheromone. Most importantly, cardboard bands with cocooned larvae must be removed at some point in time to prevent the emergence of next-generation adults. This procedure is prohibitively expensive. An alternative approach entails the development of artificial pupation sites that are readily applied to tree trunks, kill attracted larvae by means of desiccation, mechanical injury, or pathogen exposure, and thus eliminate the need for removal of such sites.
10.3 Literature cited


