

DOES PHEROMONE-BASED AGGREGATION OF CODLING MOTH LARVAE HELP PROCURE FUTURE MATES?

BRUCE DUTHIE,¹ GERHARD GRIES,^{1,*} REGINE GRIES,¹
CHRISTIAN KRUPKE,² and SHANNON DERKSEN¹

¹*Centre for Environmental Biology
Department of Biological Sciences
Simon Fraser University
Burnaby, British Columbia V5A 1S6, Canada*

(Received May 23, 2002; accepted October 16, 2002)

Abstract—In field and laboratory bioassay experiments, we show that larvae of the codling moth, *Cydia pomonella*, cocoon in aggregations. This aggregation behavior of fifth-instar larvae prior to pupation and arrestment of eclosed adult males by mature female pupae seems to allow mating as soon as an adult female ecloses. This synchronous timing is realized because foraging fifth-instar are attracted by cocoon-spinning larvae and prepupae, but not by pupae, and because male pupae develop faster than female pupae. Eclosed males are arrested by sex pheromone that disseminates from female pupae even before adult females eclose. Communication in *C. pomonella* within and among developmental stages (larva–larva and pupa–adult, respectively) may be a strategy to procure mates. If so, our data add to current knowledge that attraction of mates in insects relies on communication among adults, or pupae and adults.

Key Words—*Cydia pomonella*, larvae, pupae, aggregation behavior, aggregation pheromone, sex pheromone, mate attraction, delayed mating, mating strategy, protandry, fitness.

INTRODUCTION

Rapid acquisition of mates in short-lived adult moths may have positive fitness consequences on females or males. In gypsy moths, *Lymantria dispar*, delayed mating of females reduced the number of eggs deposited and egg viability. Moreover, with increasing age, females were less likely to mate or to receive a full

* To whom correspondence should be addressed. E-mail: gries@sfu.ca

²Present address: Tree Fruit Research and Extension Center, Washington State University, Wenatchee, Washington, 98801.

complement of sperm than females exposed to males within the first few days after eclosion (Proshold, 1996). In beet armyworms, *Spodoptera exigua*, delayed mating by females decreased fecundity and fertility, and delayed mating by males decreased the number of spermatophores they transferred to females (Rogers and Marti, 1996). In polygamous codling moths, *Cydia pomonella*, mean fecundity, fertility, and net reproductive rate of females mated 4 days after eclosion were significantly lower than for females mated on the day of eclosion or 2 days later (Vickers, 1997). Male *C. pomonella* are able to produce up to five spermatophores over the course of their adult life (Howell et al., 1978; Howell, 1988). This leads to scramble competition among males, defined by early search for and swift location of mates (Anderson and Iwasa, 1996).

For these moth species, there may be strong selection for mechanisms that ensure the presence of potential mates at the time of eclosion or for mechanisms that facilitate rapid location of mates following eclosion. Such mechanisms would facilitate rapid mating, thereby optimizing fitness for one or both mates. One such mechanism is the attraction of mate-seeking males by mature female pupae, as reported in some butterflies (Dienert et al., 1994). An alternative mechanism may involve larval aggregation behavior prior to pupation. Adults eclosing from such aggregations would immediately be near potential mates.

Aggregations of cocooning *C. pomonella* larvae reared in a laboratory and in open-fluted corrugated cardboard bands wrapped around trunks of apple trees, *Prunus malus*, provided evidence for aggregation behavior in *C. pomonella* larvae. Here we demonstrate experimentally that *C. pomonella* larvae cocoon in aggregations, and we present experimental data strongly suggesting that these larval aggregations are part of an intricate reproductive strategy to procure mates and enhance fitness.

METHODS AND MATERIALS

Experimental Insects. Larvae were shipped from the Sterile Insect Release Program in Osoyoos, British Columbia, Canada. Final instars were removed from the diet and kept in individual Petri dishes (5.5 cm diam.) for 6 hr at 22°C under a photoregime of 16L:8D. The sex of all larvae bioassayed or employed as test stimuli was determined following pupation. Female and male pupae were distinguished by the presence of three and four dark bands, respectively, on the tip of the wing pad (Petterson, 1965).

Acquisition of Larvae- and Pupa-Derived Volatiles. To obtain larvae-derived volatiles, 40 fifth instars were placed in a horizontal Pyrex glass chamber (30 × 1.2 cm). An empty chamber served as a control. Water aspirators were used to draw humidified, charcoal-filtered air at a rate of 80 ml/min through each chamber and through a glass column (6 × 30 mm) filled with Porapak Q (50–80 mesh, Waters Associates, Inc., Milford, Massachusetts, USA). After 48 hr, volatiles were

desorbed with 2.5 ml of redistilled pentane plus ether (95:5). Larvae cocooning inside the glass chamber were rinsed with 30 ml of pentane plus ether (95:5). Extracts were concentrated under a fine stream of nitrogen.

To obtain pupae-derived volatiles, 40 newly formed female or male pupae were placed separately in Pyrex glass tubes (30 × 0.7 cm) maintained at 23°C and an airflow of 40 ml/min. During days 0–2, 3–5, 6–8, 9–10, 11, and 12, volatiles were captured with Porapak Q and desorbed as above. In addition, 12-day-old male and female pupae were rinsed with pentane plus ether (95:5).

Experiment 1: Evidence for Aggregation by Larvae in the Field. On August, 21, 2001, the trunks of 163 apple trees in orchards of the Tree Fruit Research and Extension Center of Washington State University (Wenatchee) were wrapped with an open-fluted, corrugated cardboard band (10 cm wide; flute width: ~ 8 mm), 20–50 cm above ground. On October 21, 2001, all bands were collected and frozen (–10°C). For each band, the position of all cocoons was measured with callipers to the nearest 0.1 mm. The null hypothesis tested was that cocoons generally (>95%) do not occur in aggregations.

Experiment 2: Evidence for Aggregation by Larvae in the Laboratory. Ten fifth-instar larvae were placed in the center of each of 10 plastic Petri dishes (14 cm diam.). After 24 hr, cocooning larvae were recorded as solitary, aggregates of 2–5, and aggregates of >5.

Experiment 3: Aggregation to Arrest Males. Each replicate consisted of six treatments (Table 1). In pairs of pupae, the pupa mentioned first was one day older and typically eclosed earlier, allowing us to record the adult's response to the presence of a conspecific pupa. Eclosion, arrestment, and departure of male and female adults were recorded from each jar (Figure 1A) at 12-hr intervals. Because the experiment tested the hypothesis that the presence of a mature female pupa arrests an eclosed adult male, replicates with paired pupae were terminated when both adults had eclosed. In treatments with a single male or female pupa, the replicate was terminated when the eclosed male or female had departed.

Experiments 4–6: Time of Aggregation. In olfactometers (Figure 1B) (Pierce et al., 1981), potential pupation sites (Table 1) were randomly assigned to one of two vials. For each replicate, a fifth instar was released in the center of the olfactometer and its pupation site recorded. All experiments were conducted in an environmental chamber at 22°C and a photoregime of 16L:8D.

Experiment 7: Timing of Pupal Development. Each of 60 fifth instars was placed in a Petri dish (5 cm diam.) at 23°C to record duration of the pupation period.

Experiments 8 and 9: Pheromone-Based Larval Aggregation. The same protocol was employed as in experiments 4–6, except that perforated plastic inserts in vials (Figure 1C) prevented physical contact of foraging larvae with test stimuli, which consisted of cocooning larvae (experiment 8) or collected volatiles of cocooning larvae (experiment 9) (Table 1). In experiment 9, aliquots

TABLE 1. SUMMARY OF EXPERIMENTS CONDUCTED WITH *C. pomonella*

Exp.	Title	Design or stimuli	Replicates (N)
1	Aggregation by larvae in the field	Open-fluted, corrugated cardboard band on tree trunk	163
2	Aggregation by larvae in the laboratory	10 larvae in Petri dish	10
3	Stimulus-dependent arrestment of eclosed adults	♂ + ♂ pupae	12
		♂ + ♀ pupae	12
		♂ pupa	12
		♀ + ♀ pupae	12
		♀ + ♂ pupae	12
		♀ pupa	12
Test stimuli in choice experiments ^a			
4	Stimulus-dependent attraction of larva	Cardboard + cocooning larva Cardboard	33
5	Stimulus-dependent attraction of larva	Cardboard + prepupa in cocoon Cardboard	45
6	Stimulus-dependent attraction of larva	Cardboard + pupa in cocoon Cardboard	28
7	Duration of pupation period	1 fifth-instar in Petri dish	60
Test stimuli in choice Experiments ^b			
8	Stimulus-dependent attraction of larva	Cardboard + cocooning larva Cardboard	28
9 ^c	Stimulus-dependent attraction of larva	Cotton wick + volatiles Cardboard from cocooning larvae	26
10 ^d	GC-EAD analyses of pupa-derived volatiles	Volatiles from ♀ or ♂ pupae	4

^a Test stimuli could be contacted by the foraging larva.

^b Perforated plastic inserts in vials (Figure 1C) prevented the foraging larva from contacting test stimuli.

^c Aliquots tested were equivalent to a solvent rinse from 40 cocoon-spinning larvae plus 19 volatile hour equivalents of cocooning larvae [collected with Porapak Q and desorbed with pentane-ether (95:5)]; each treatment and control stimulus received 25 μ l of solvent.

^d Aliquots analyzed by GC-EAD were equivalent to solvent rinses from 18 female pupae, and volatiles from 35 male or female pupae collected with Porapak Q for 1 hr and desorbed with pentane-ether (95:5).

of both Porapak Q volatile extract and solvent rinses of cocooning larvae were released from cotton wicks to test their attractiveness *versus* that of a solvent control.

Experiment 10: GC-EAD Analyses of Pupa-Derived Volatiles. Aliquots of Porapak Q trapped volatile extracts from, and solvent rinse extracts of, 14-day-old male and female pupae were analyzed by coupled gas chromatographic-electroantennographic detection (GC-EAD) (Arn et al., 1975), employing a

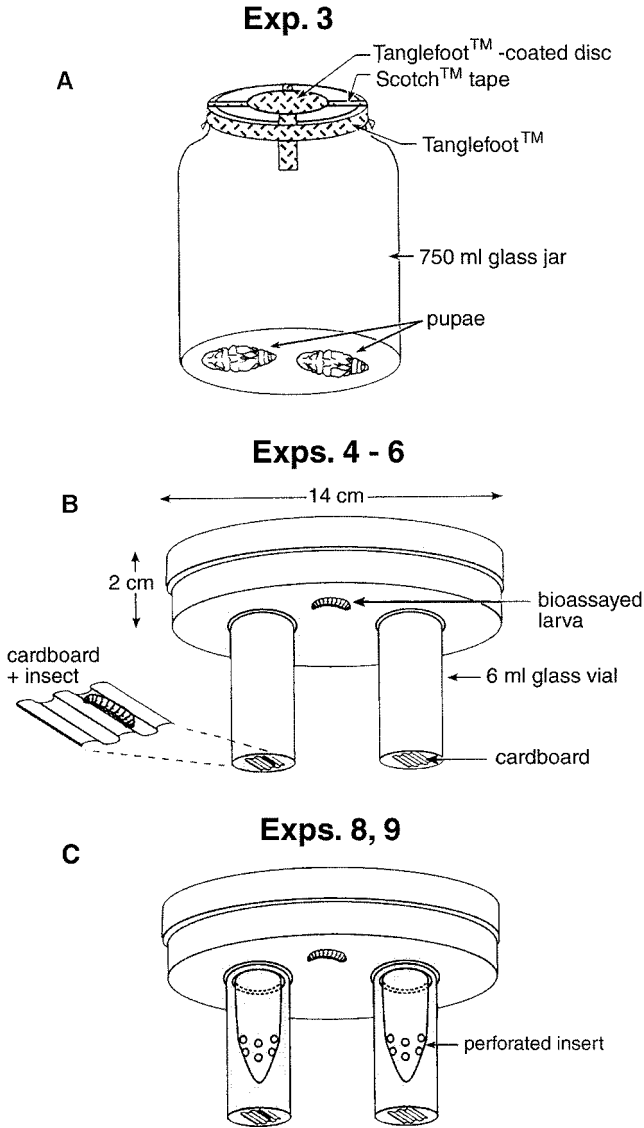


FIG. 1. Bioassay devices. (A) Glass jar modified such that eclosed *Cydia pomonella* adults could depart but not reenter. (B) Two-choice olfactometer (adapted from Pierce et al., 1981). (C) Two-choice olfactometer with perforated plastic inserts in glass vials that prevented the bioassayed *C. pomonella* larva from contracting test stimuli.

Hewlett Packard (HP) 5890A gas chromatograph equipped with a fused silica column (30 m \times 0.25 or 0.32 mm ID) coated with DB-5, DB-23, or DB-210 (J&W Scientific, Folsom, California, USA) or SP-1000 (Supelco, Bellefonte, Pennsylvania, USA). For GC-EAD recordings, an antenna was gently removed from a male's head and suspended between glass capillary electrodes filled with insect Ringer's solution (Staddon and Everton, 1980). Identical retention times of antennal responses to synthetic and natural (E,E)-8,10-dodecadien-1-0 on each of four GC columns (see above) provided strong evidence that (E,E)-8,10-dodecadien-1-0 was produced in the late stage of female, but not male, pupae.

Statistical Analyses. The null hypothesis that *C. pomonella* cocoons do not occur in aggregations in open-fluted cardboard bands wrapped around tree trunks (experiment 1) was tested by the U^2 statistic (Watson, 1961) developed for discrete (here flute-based) data around a circle (Choulakian et al., 1994). Aggregation behavior of larvae in experiment 2, and number of larvae responding to stimuli in olfactometer bioassay experiments 4–6, 8, and 9 were analyzed with the χ^2 goodness-of-fit test using Yates' correction for continuity or Fisher's exact test to determine whether observed frequencies deviated significantly from expected frequencies (Zar, 1996). Arrestment times of newly eclosed adult moths in experiment 3 were analyzed by nonparametric analysis of variance followed by comparison of means [Bonferoni (Dunn) t -test]. The results for male and female pupation times in experiment 7 were analyzed with the paired-sample t test. In all experiments $\alpha = 0.05$.

RESULTS AND DISCUSSION

Our results show that: (1) *C. pomonella* larvae aggregate prior to pupation (2) larvae select pupation sites based on the presence and developmental stage of conspecifics (3) larval aggregations help arrest future mates, and (4) pheromones mediate both aggregation of larvae and arrestment of eclosed adults.

Aggregations of larval cocoons (Figure 2) were found in many cardboard bands and were confirmed to be statistically significant for 64 ($P \leq 0.05$) or 88 ($P \leq 0.1$) of 163 bands (Figure 3A). The null hypothesis that cocoons generally (>95%) do not occur in aggregations was rejected based on these results. Aggregations of larval cocoons were also confirmed in a laboratory experiment (Figure 3B). The adaptive significance of these aggregations of male and female larvae could be to arrest eclosing adult males and to procure future mates for females. Males that eclosed from pairs of a female plus male pupa were arrested significantly longer than males that eclosed from single or paired male pupae (Figure 3C).

Timing is of the essence in the procurement of future mates by larvae. Attraction of foraging fifth instars by cocoon-spinning larvae and prepupae, but not by pupae (Figure 4A), ensures eclosion of adults within the same relative time period.

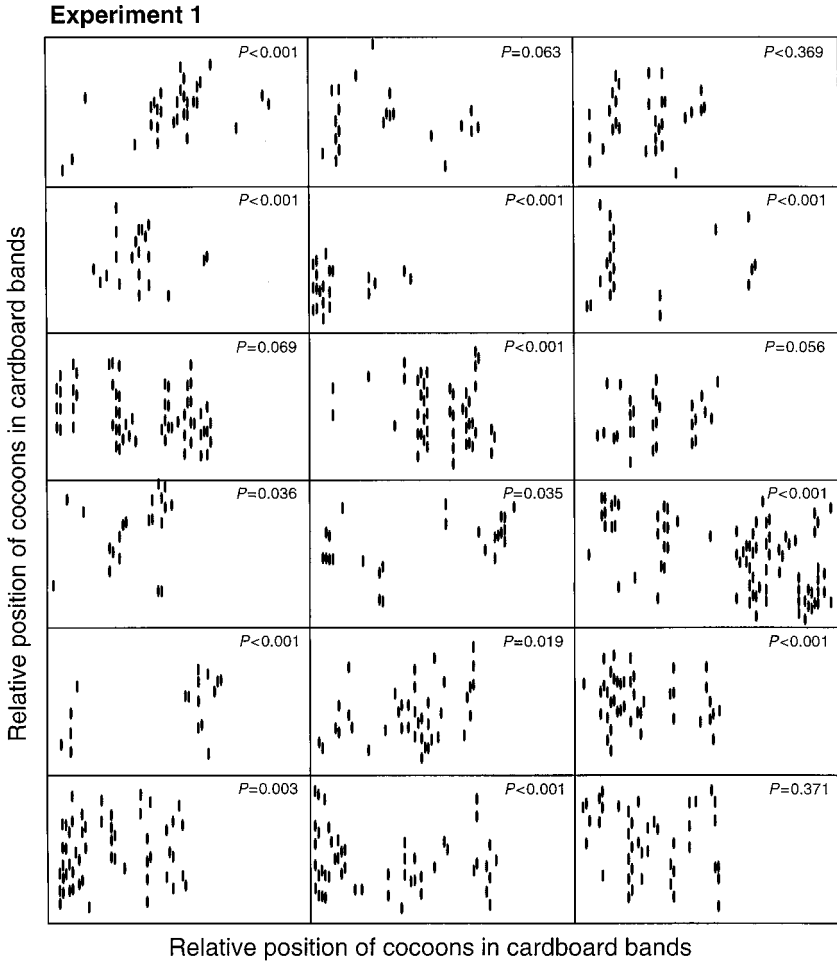


FIG. 2. Graphical presentations of relative positions of *Cydia pomonella* cocoons in 18 (of 163) open-fluted cardboard bands (10 cm wide) placed on trunks of apple trees, *Prunus malus*, in orchards of the Tree Fruit Research and Extension Centre of Washington State University, Wenatchee, August 21– October 21, 2001. In each band, $P < 0.05$ indicates aggregation of larval cocoons. Note: apparent evidence for aggregation of larval cocoons could not be confirmed statistically for each band.

Adult males eclose before females (Dolstad, 1985). A male larva, for example, being attracted to a female prepupa will pass through the pupal stage faster than the female (Figure 4B), thereby making up for the advanced development of the female prepupa. As a result, the male ecloses just in time to await eclosion of

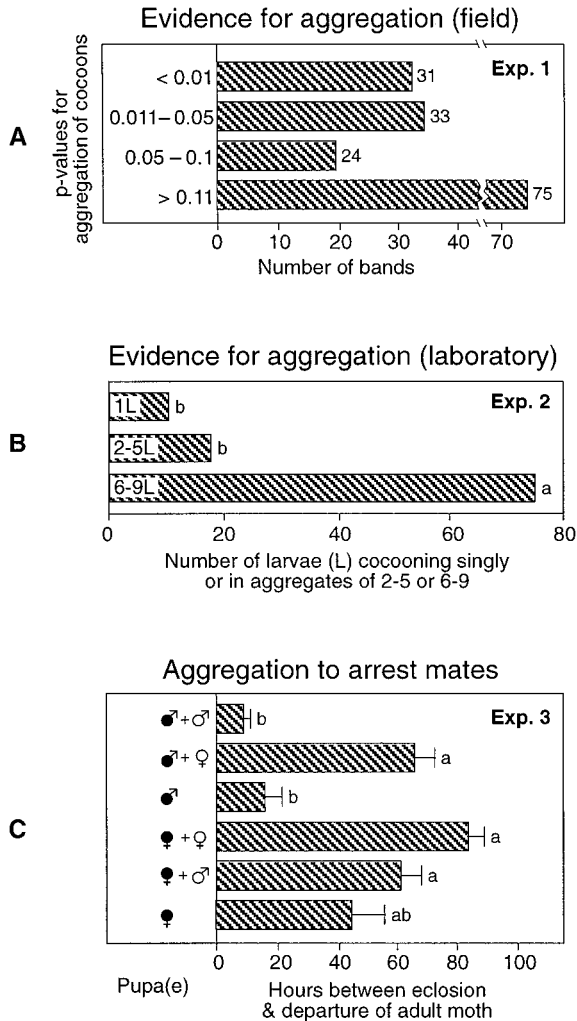


FIG. 3. (A) Number of open-fluted cardboard bands with respective levels of significance for aggregation of *Cydia pomonella* larval cocoons. (B) Number of larvae in 10 groups of 10 larvae cocooning singly or in aggregates (2–5 or >5). (C) Stimulus-dependent arrestment of eclosed adults. Solid and open symbols represent insects of identical age. In treatments of paired insects, those represented by solid symbols were one day older. Each replicate was terminated when both adults in pairs of pupae had eclosed or the adult from a single pupa had departed. Arrestment periods reported in this study may be longer than those occurring in nature, because presence or departure of the adults was recorded only every 12 hr. In each of experiments 1–3, bars with different letters are significantly different; $\alpha = 0.05$.

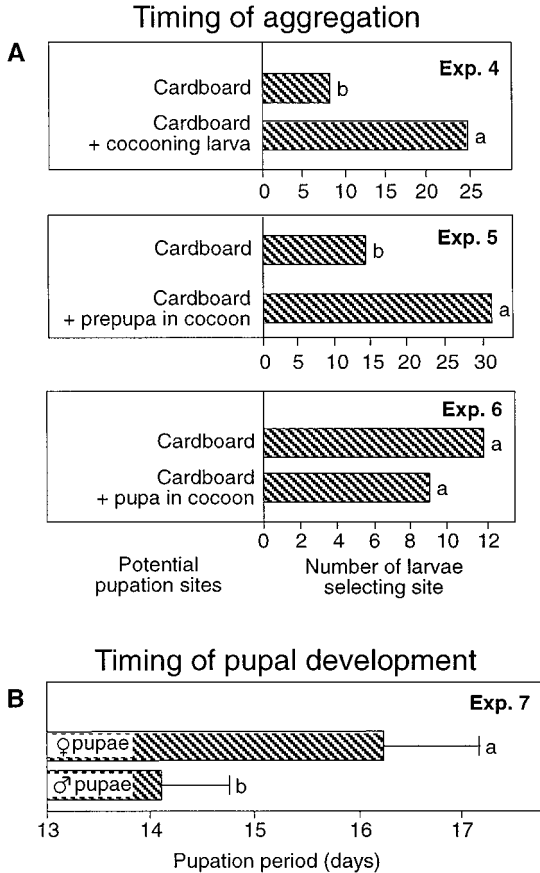


FIG. 4. (A) Selection of potential pupation sites by fifth-instar *Cydia pomonella* in olfactometer (Figure 1B) bioassay experiments in response to accessible test stimuli. (B) Average pupation periods of male and female pupae at 23°C. In each experiment, bars with different letters are significantly different; $\alpha = 0.05$.

the female. Even if the male's eclosion preceded that of a female by >24 hr, the arrestment signal from the female pupa (Figures 3C and 5B) should prevent the adult male from departing. The female's tactic of remaining at the site of eclosion for a significant time (Figure 3C) should increase her chance of securing a mate. While she is calling for a foraging male, a male from the pupal aggregate may eclose and mate with her.

Airborne pheromones mediate both aggregation of larvae (Figures 2 and 3A,B) and arrestment of adult males by female pupae (Figure 3C). These conclusions arise from the findings that: (1) foraging fifth instars were attracted to

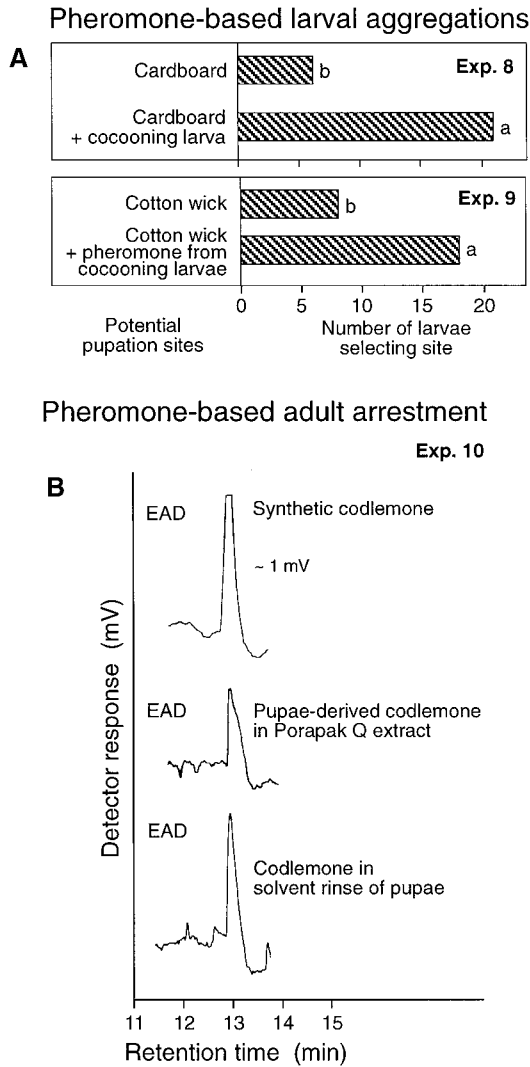


FIG. 5. (A) Selection of potential pupation sites by fifth-instar *Cydia pomonella* in olfactometer (Figure 1C) bioassay experiments in response to test stimuli. Perforated plastic inserts prevented contact with test stimuli. (B) Electroantennographic detector (EAD: male *C. pomonella* antenna) responses to 10 pg of synthetic pheromone (*E,E*)-8,10-dodecadienol (top), 35 volatile hour equivalents of female pupae (middle), and to solvent rinses from 18 female pupae (bottom). Chromatography: Hewlett Packard 5890A gas chromatograph equipped with a DB-23 coated column (30 m \times 0.25 mm ID); linear flow velocity of carrier gas: 35 cm/sec; injector temperature: 280°C; temperature program: 1 min at 100°C, 10°C/min to 200°C.

cocoon-spinning larvae or their volatile extracts even when physical contact with the volatile source was prevented (Figure 5A), and (2) male antennae in GC-EAD analyses of volatile extracts from mature pupae detected codlemone, (*E,E*)-8,10-dodecadienol (Figure 5B), the known female-produced sex pheromone (Roelofs et al., 1971). Codlemone was not detectable in extract analyses of early pupal stages.

Our findings represent evidence of a fail-safe mechanism of mate procurement. Should attraction of male larvae by female larvae or prepupae (Figures 4A and 5A) and arrestment of eclosed adult males by female pupae (Figure 3C) fail, eclosed females can still resort to calling behavior to attract foraging males. Procurement of future mates by larvae would be advantageous to both sexes. Females that mated just after eclosion laid more eggs and produced more females than those that mated several days later (Vickers, 1997). Males, in turn, pass on their genes even before they commence foraging, which is adaptive should they perish during foraging or fail to locate a calling female. Males may also secure mating with several females, select the most profitable female(s), and/or gain sperm precedence (Bonduriansky, 2001).

Communication in *C. pomonella* within and among developmental stages, larva–larva (Figures 4A and 5A) and pupa–adult (Figures 3C, and 5B), may be a strategy to procure mates. If so, our data add to current knowledge that attraction of mates in insects relies on communication among adults, or pupae and adults (Phelan, 1997). They may indicate that communication in *C. pomonella*, and possibly other insects, is more intricate than previously realized.

Acknowledgments—We thank J. H. Borden and M. Winston for their discussions; B. Crespi and M. Winston for review of the manuscript; H. Pierce, Jr., for his assistance in volatile collections; S. Takács for technical advice; B. Mircioiu, L. Mircioiu, and M. Chatterton for technical assistance; E. Carefoot for preparation of figures; M. A. Stephens for statistical analyses of cocoon distributions in cardboard bands; V. Pleasance for shipment of codling moth larvae; and L. Duthie for support and encouragement. The research was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to G.G.

REFERENCES

- ANDERSON, M. and IWASA, Y. 1996. Sexual selection. *Trends Ecol. Evol.* 11:53–58.
- ARN, H., STÄDLER, E., and RAUSCHER, S. 1975. The electroantennographic detector—a selective and sensitive tool in the gas chromatographic analysis of insect pheromones. *Z. Naturforsch.* 30c:722–725.
- BONDURIANSKY, R. 2001. The evolution of male mate choice in insects: a synthesis of ideas and evidence. *Biol. Rev.* 76:305–339.
- CHOUKAKIAN, V., LOCKHART, R. A., and STEPHENS, M. A. 1994. Cramér-von Mises statistics for discrete distributions. *Can. J. Stati.* 22:125–137.
- DIENERT, E. T., LOGINO, J. T., and GILBERT, L. E. 1994. Mate competition in butterflies. *Nature* 370:23.
- DOLSTAD, K. D. 1985. Biology and control of the codling moth in the Pacific Northwest. Master of Pest Management thesis. Simon Fraser University, Burnaby, British Columbia, Canada.

- HOWELL, J. F. 1988. Spermatophore number in the codling moth, *Cydia pomonella* L. (Lepidoptera: Olethreutidae). *Can. Entomol.* 120:701–710.
- HOWELL, J. F., HUTT, R. B., and HILL, W. B. 1978. Codling moth: mating behavior in the laboratory. *Ann. Entomol. Soc. Am.* 71:891–895.
- PETTERSON, D. M. 1965. A quick method for sex determination of codling moth pupae. *J. Econ. Entomol.* 58:579.
- PHELAN, L. P. 1997. Evolution of mate-signalling in moths: phylogenetic considerations and predictions from the asymmetric tracking hypothesis, in J. C. Choe and B. J. Crespi (eds.). *Mating Systems in Insects and Arachnids*. Cambridge University Press, Cambridge.
- PIERCE, A. M., BORDEN, J. H., and OEHLISCHLAGER, A. C. 1981. Olfactory response to beetle-produced volatiles and host-food attractants by *Oryzaephilus surinamensis* and *O. mercator*. *Can. J. Zool.* 59:1980–1990.
- PROSHOLD, F. I. 1996. Reproductive capacity of laboratory-reared gypsy moths (Lepidoptera: Lymantriidae): Effect of age of female at time of mating. *J. Econ. Entomol.* 89:337–342.
- ROELOFS, W., COMEAU, A., HILL, A., and MILICEVIC, G. 1971. Sex attractant of the codling moth: characterization with electroantennogram technique. *Science* 174:297–299.
- ROGERS, C. E. and MARTI, O. G., JR. 1996. Beet armyworm (Lepidoptera: Noctuidae): effect of age at first mating on reproductive potential. *Fla. Entomol.* 79:343–353.
- STADDON, B. W. and EVERTON, I. J. 1980. Haemolymph of milkweed bug (*Oncopeltus fasciatus* (Heteroptera: Lygaeidae): Inorganic constituents and amino acids. *Comp. Biochem. Physiol.* 65:371–374.
- VICKERS, R. A. 1997. The effect of delayed mating on oviposition pattern, fecundity, and fertility in codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae). *Aust. J. Entomol.* 36:179–182.
- WATSON, G. S. 1961. Goodness-of-fit tests on a circle. *Biometrika* 48 (1 and 2):109–114.
- ZAR, J. H. 1996 *Biostatistical Analysis*. Prentice-Hall, Englewood Cliffs, New Jersey.