Reduced antennal sensitivity, behavioural response, and attraction of male codling moths, *Cydia pomonella*, to their pheromone (*E*,*E*)-8,10-dodecadien-1-ol following various pre-exposure regimes

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Abstract

The effects of pre-exposing male codling moths, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), to their pheromone (*E*,*E*)-8,10-dodecadien-1-ol (codlemone), in static and moving air, under laboratory and field conditions, on subsequent antennal sensitivity, behavioural responsiveness, and attraction to codlemone were investigated. In flight tunnel experiments, the percentage of moths wing fanning and taking flight were mostly unaffected, but upwind flight to, and contact with, pheromone sources known to elicit responses of both were shown to depend on the intensity and duration of previous exposure to codlemone and recovery time between exposure and assessment. Ten to 30-min pre-exposures to codlemone in static air (≈ 35 μg l^{-1}) not only caused a 99% reduction in attraction, but also significantly reduced electroantennogram response to codlemone. Recovery of full antennal sensitivity to codlemone took more than 1 h, but recovery of attraction took over 4 h, suggesting that habituation is also partially involved in reduced behavioural responsiveness following pre-exposure. Seventy-five min exposures to codlemone in moving air (5–10 cm s^{-1}) at rates of 0.9, 4.5, and 18 μg h^{-1} from Celcon fibres caused 75, 86, and 99% disruption, respectively. However, 30–34-h exposure of caged moths to air moving through an orchard treated with 1000 Isomate-C® dispensers ha^{-1} releasing approximately 20 μg h^{-1} per dispenser during tests, had no impact on moth response in flight-tunnel assays 30 min after removal from the orchard. In this treated orchard, catches of free-flying moths in pheromone-baited traps were completely inhibited. If observed mechanisms such as long-lasting antennal adaptation or habituation of the central nervous system contribute to the disruption of pheromone communication among codling moths under field conditions, it seems unlikely that they occur following exposure to the average atmospheric concentrations of codlemone. For these effects to be important, codling moths may require close contact with pheromone sources for extended periods, or repeated close encounters.

Introduction

In western North America, the use of pheromone-based mating disruption for control of the codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), is replacing use of broad-spectrum insecticides as a pest management paradigm in pome-fruit production (Calkins, 1998; Gut & Brunner, 1998). Hand-applied pheromone dispensing systems like Isomate® 'twist-tie ropes' have captured the largest share of this pheromone-management market (Howell, 1992; Judd et al., 1996; Thomson et al., 2001), but there is increasing interest in using alternative pheromone formulations and delivery systems (Hall & Marrs, 1989; Shorey & Gerber, 1996). The implementation of different pheromone-delivery systems in this established pome-fruit pest management program requires practical decisions about the importance of pheromone blends, release rates, and densities of dispensers, which vary across delivery systems and potentially impact the mode of action

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and success of communication disruption (Weatherston, 1990).

Historically, the understanding to make these decisions has come from empirical field studies, in part, because there are few laboratory procedures available to speed the process (Minks & Cardé, 1988). To date, this approach has not answered a fundamental question: what is it that makes an effective pheromone-based mating-disruption system? Biased to some extent by an emphasis on the chemistry of multicomponent pheromone blends and their central role in the orientation process of moths, researchers have focused on the role of confusion by false-trail following as a primary disruption mechanism (Bartell, 1982), but one for which there is still little quantitative data (Rothschild, 1981; Novak & Roelofs, 1985; Schmitz et al., 1995, 1997; Evenden et al., 1999a,b,c). One argument often forwarded by pheromone scientists to explain the failure of mating disruption is that use of suboptimal synthetic blends compromises control by eliciting submaximal orientation (Minks & Cardé, 1988; Witzgall et al., 1999). However, a body of literature indicates that any false-trail following that does not act in conjunction with some other mechanism is likely to be an ineffective disruption tool (Kuenen & Baker, 1981; Sanders, 1982, 1985, 1995, 1996; Mafra-Neto & Baker, 1996; Sanders & Lucuik, 1996).

If mechanisms for disrupting communication were studied in depth, and the relative importance of each better understood, pest management practitioners might be in a position to compare disruption products in some standardized way. In this context, while various commercial pheromone-based mating-disruption systems are available to control codling moth in North America, little is known about how they actually work, and some scientists have questioned if they do at all (Knight, 1996). While several plausible mechanisms for disrupting pheromone communication have been proposed (Bartell, 1982) and expounded on (Cardé, 1990), postulating these mechanisms has been easier than finding definitive proof that they actually occur. More importantly, it is difficult to say which of these mechanisms, or groups of mechanisms, are most effective when invoked and therefore, those that should be defining the design of pheromone systems. Even in well-studied species like the pink bollworm, where there is compelling evidence of multiple mechanisms (Cardé et al., 1998), it is not known which of these are most effective.

Scientists have done a better job of describing how communication disruption might work than explaining to engineers what they should be designing in order to achieve effective results. Perhaps this is because much of what is known about the mechanisms of communication disruption has been gleaned from commercial mating-disruption programmes. The interpretation of these field results is difficult when the mechanisms acting in them are not clearly understood, especially since the mechanisms can vary with the pheromone products being tested (Weatherston, 1990). Making inferences about the importance of particular communication disruption mechanisms because pheromone mating-disruption products have succeeded or failed as crop-protection tools could lead to false conclusions about the relative importance, or lack of importance, of certain mechanisms. Many ecological factors can undermine these behavioural control efforts. Rumbo & Vickers (1997) pointed out that a concerted effort to better understand mechanisms of communication disruption using experiments specifically designed for this purpose is somewhat lacking. This observation remains true, except in a few species (Cardé et al., 1998; Svensson et al., 2001).

Kuenen & Baker (1981) suggested that repeated false-trail following that potentially leads to pulsed exposures to high concentrations of pheromone might provide more a effective disruption of communication by causing longer term habituation of various olfactory processes. A plethora of supporting studies (Bartell & Roelofs, 1973; Bartell & Lawrence, 1976, 1977a,b; Linn & Roelofs, 1981; Sanders, 1985; Figueredo & Baker, 1992; Mafra-Neto & Baker, 1996; Sanders & Lucuik, 1996; Rumbo & Vickers, 1997; Daly & Figueredo, 2000) has shown that the pre-exposure of adult male Lepidoptera to pheromone sources causes significant changes in their behavioural responsiveness to subsequent exposures. Recently, Stelinski et al. (2003a,b) have hypothesized that among tortricid moths at least, long-term adaptation of antennal pheromone receptors and its potential impact on habituation of the central nervous system may play an important role in determining the success of mating-disruption programs for some species.

Experiments reported here are directed towards understanding: (1) the potential role of neurophysiological effects in disrupting pheromone communication in the codling moth; (2) the extent to which previous exposure to their major pheromone component (*E*,*E*)-8,10-dodecadien-1-ol (codlemone) causes adaptation of olfactory receptors, and/or habituation of the central nervous system, resulting in the moths' inability to locate sources of pheromone, and (3) how the mode of pheromone presentation might impact any of these neurological effects.

Materials and methods

Laboratory insect culture

The codling moths used in these experiments came from the mass-rearing colony at the Okanagan-Kootenay Sterile Insect Release Program rearing facility in Osoyoos, British Columbia (BC), Canada (Dyck & Gardiner, 1992). Trays of artificial diet (Brinton et al., 1969) with developing

larvae were held in environmental chambers at 25 °C under a L16:D8 photoperiod. As they developed, the pupae were removed from the diet, sexed, and placed individually in 150-ml plastic cups provided with a wet cotton wick until the adults eclosed. Male and female moths were isolated from each other in separate environmental chambers, each maintained at 25 °C, 65% r.h., and L16:D8 photoperiod.

Flight tunnel description

A pushing-type flight tunnel based on the design of Miller & Roelofs (1978) and described in detail by Evenden et al. (2000) was used to assess the behavioural responsiveness of naïve and pre-exposed male moths to pheromone sources in clean air. Air was pushed through the tunnel flight section at 35–40 cm s^{-1}, and the pheromone plume was exhausted from the tunnel with a centrally located variable speed fan that vented outside the building. The 2.45 m long × 1 m high tunnel flight section, made of clear Lexan® bent in a semicylindrical shape, was illuminated uniformly by overhead white light provided by six 25 W incandescent bulbs (Philips 120 V Inside Frost, Philips Electronics Ltd, Scarborough, Ontario, Canada) housed in a light box constructed from white Coroplast® plastic (Boyds, Kelowna, BC, Canada) positioned 13 cm above the tunnel. Mean light intensity at the point of male release and plume height was 1.8 lux (Minolta Auto Meter IVF). The floor of the tunnel flight section had an irregular pattern of 10-cm diameter dark blue solid circles on a white background.

Pheromone lures and pre-exposure sources

In flight tunnel assays, the moths were presented with either of two types of pheromone lures that our research (unpubl.) has shown to elicit similar responses in the flight tunnel. Red rubber septa (Aldrich Chemical Company Inc., Milwaukee, Wisconsin, USA) were loaded with 200 µl of dichloromethane containing 10 µg of codlemone (99% isomeric and chemical purity, Shin-etsu, Fine Chemicals Division, Tokyo, Japan), air dried for ca. 18 h at 23 °C in a fume hood, and stored in sealed jars at –25 °C until use. Paper lures made from 3 × 3 cm folded strips of Whatman no. 5 filter paper were treated with 50 µl solutions of dichloromethane containing 2.5 µg of codlemone, air dried at room temperature for 30 min in a fume hood, sealed in glass jars, and stored at –25 °C until use. Unless otherwise stated, all pre-exposed moths were presented with varying amounts of codlemone dissolved in dichloromethane applied in 100 µl aliquots to 3-cm diameter Whatman no. 5 filter paper discs that had been aged at room temperature for 20 min in a fume hood and stored at –25 °C in sealed jars until needed for experiments.

General moth handling procedures

For each flight tunnel experiment, 24- to 96-h-old male moths were placed in a lit, 2.5 °C chamber, 3 h before scotophase and cooled for 20 min. For each replicate of a given experiment the same fixed proportion of different-aged moths was used for treatment and control groups to reduce variation across replicates. Groups of 14–20 immobilized moths were placed inside cylindrical (12.5 cm tall × 6.5 cm diameter) stainless steel wire mesh cages. Caged moths were re-warmed at room temperature in a fume hood and 60 min before scotophase they were placed inside 1-l glass mason jars on stainless steel mesh platforms set 3 cm off the bottom of the jar above a Teflon®-coated magnetic stir bar. Five min before placing the cages in jars, a solvent- or codlemone-impregnated filter paper disc was placed on the platform beneath the 1-cm recessed bottom of the cage which prevented contact of the moth with the filter paper. Jars were sealed with Teflon®-lined metal screw top lids and placed on an electric stir plate that mixed solvent- or codlemone-treated air throughout the exposure period. New pre-exposure filter papers were used for each exposure replicate.

After pre-exposure at room temperature, caged moths were chilled for ca. 10 min and immobilized moths were placed in release cages and transferred to the flight-tunnel room 15 min before scotophase. Inside the flight-tunnel room moths were placed inside a clear Plexiglass® holding unit where a fan constantly blew charcoal-filtered air over them, preventing any further exposure to pheromone before testing. An exhaust hood in the tunnel room pulled room air over stored pheromone sources and exhausted it outside the building. Flights usually commenced ca. 15 min after the onset of scotophase. Total time between pre-exposure and the flight of the first and last moths was usually 40 and 80 min, respectively.

Single moth bioassay

Filter paper pheromone lures were hung 48 cm above the tunnel floor and 34 cm from its upwind end in a wire loop suspended from a vertical metal ring stand. Cylindrical (1.5 cm high × 3 cm diameter) wire mesh release cages with sheet-metal lids containing individual moths were introduced into the pheromone plume on a mobile release device through a port in the side of the tunnel, and pushed into the plume on rails by a lever operated from the outside. After 10 s in the plume, a fishing line with Velcro® attachment to the release device lid was pulled from outside the tunnel, and the moths were released. A timer was set for 1 min and individual moth responses were scored as yes or no for the following behaviours: (1) wing fanning, (2) take-off flight, (3) locking onto plume with upwind flight, and (4) source contact. Fifteen to 20 naïve

and pre-exposed moths were flown individually in random order on each of several days. Each day's entire flight session lasted no more than 70 min and lures were changed every 20 min to maintain consistent pheromone delivery. After each day's session all metal flight-tunnel components were washed in acetone and heated for ca. 12 h at 200 °C. The Velcro® attachments on the release-device lids were replaced for each session.

Group moth bioassay

In group assays the behavioural measure of pre-exposure effects was the percentage of moths being trapped in the flight tunnel. All group-assay moths were dusted lightly with differently coloured Day-Glo® Daylight Fluorescent Powders (Switzer Brothers Inc., Cleveland, OH, USA) before being placed inside mesh cages during the pre-exposure phase. These marks allowed for the simultaneous release of moths receiving different treatments or the release of moths in rapid succession without having to remove untrapped moths from the flight tunnel between tests.

Pheromone lures were pinned inside cylindrical aluminium traps (13 cm long × 11 cm diameter) lined with clear 1 mm thick polyester, coated internally with STP Oil Treatment (First Brands Corporation, Scarborough, ON, Canada) to trap alighting moths. One lure-baited trap was hung from a ring stand at the upwind end of the tunnel flight section (48 cm above the tunnel floor, 34 cm downwind from the upwind screen surface). Moths were introduced into the pheromone plume at lure height, 44 cm above the tunnel floor and 150 cm downwind from lures, on the mobile release device. Moths were exposed to the pheromone plume for ca. 10 s before the externally operated line was used to lift the lids of wire mesh release cages (2 cm high × 9 cm diameter), allowing males to exit. Each flight period with each group of moths lasted 10 min, after which the pheromone lure and trap liner were replaced and another group of moths was introduced to the flight tunnel. The number of males caught in pheromone-baited traps was recorded and a UV light was used to illuminate their fluorescent mark. Only moths bearing the corresponding mark of a specific flight were used in the analyses. After each flight day, all cages, lids and reusable trap parts were rinsed with acetone and heated for ca. 12 h at 200 °C to remove all possible pheromone contamination.

Pheromone concentration in pre-exposure chambers

Thermogravimetric analysis (TGA) was used to estimate the airborne concentration of codlemone that volatilized from treated filter papers in pre-exposure jars. TGA involves using a computer-controlled precision balance that constantly weighs a known mass for a set time while it is held at constant temperature in a semi-enclosed precision oven (Mettler Toledo, Model TGA/SDJA851). Performing TGA on pure dichloromethane followed by TGA of codlemone in solutions of dichloromethane, and subtracting the two curves it was possible to generate an equation from the resulting plot of declining mass against time and predict the rate of codlemone volatilization for given test conditions. By subtracting amounts of codlemone that adsorb on the inside wall of glass jars it was possible to estimate the total airborne concentration of codlemone present in mason jars throughout pre-exposure periods for given solute concentrations applied to filter paper. Within 5–10 min of removing the pheromone-impregnated filter paper from exposure jars, the inside walls were rinsed several times with methanol (2 ml each rinse) to remove any codlemone adsorbed onto glass. Methanol washes were concentrated under a stream of N gas, and analyzed by gas chromatography using a Hewlett-Packard Model 6890 instrument with flame ionization detection on a DB-5 capillary column (split-less injection, 30 m × 0.32 mm ID) programmed from 50 °C (hold 1 min), ramped 10 °C min^{-1} to 230 °C, and operated with He carrier gas. Dodecanol was used as an internal standard.

To reduce the adsorption of codlemone onto glass surfaces, all pre-exposure jars were pre-rinsed internally with 500 ml of 1 M NaOH for 24 h on a revolving roller unit and silanized with trimethylchlorosilane (TMCS), which reduces bonding sites. Silanization involved pre-heating the glass jars to 200 °C for 24 h and then rinsing them internally with 10 ml of TMCS in toluene (10: 90, vol/vol) for an additional 24 h.

Experiments 1–4: pre-exposure dose

Three experiments were performed using single-moth assays to determine at what point in a behavioural sequence any effects of pre-exposure to codlemone might be observed, and to identify an appropriate behavioural response with which to score the effects of pre-exposure. In three separate experiments (Experiments 1–3), groups of 20 moths were pre-exposed in jars with 100, 500, or 1000 μg of codlemone for 30-min periods, respectively. The responsiveness of moths pre-exposed to these treatments was compared pair-wise with groups of 20 control moths exposed for 30 min to solvent alone. In Experiment 4, a group-moth assay was employed to test the effects of the pre-exposure dose. Three separate groups of 20 moths were pre-exposed simultaneously to either 5, 50, or 500 μg of codlemone for 30-min periods, respectively. A fourth group of 20 moths, exposed to solvent alone for 30 min, served as the control group. On each of 4 days, these four groups of treated and control moths were presented separately at 20-min intervals into

the flight tunnel using a 4 × 4 Latin-Square randomization. This Latin-Square design ensured that any variation resulting from replication on separate days and assay times within a day was blocked (Zar, 1984).

Following these first four experiments, it was determined that a group-assay technique based on percentage capture of groups of moths in pheromone-baited traps following pre-exposure could be as useful and more rapid than single-moth assays measuring source contact. The group-assay protocol was therefore adopted for all subsequent tests.

Experiments 5–6: pre-exposure duration

Two experiments were performed to determine how long an exposure to codlemone was required to see a behavioural effect. In Experiment 5, four separate groups of 14–20 moths were exposed to 500 μg of codlemone for 2, 4, 6, or 8 min and all pre-exposure treatments ended at the same time by adjusting the respective start times for pre-exposure. A fifth group of 12–20 moths, exposed to solvent for 8 min, served as the control group. Following a similar procedure to that used in Expt 4, groups of treated or control moths were introduced one at a time into the flight tunnel on each of five separate days following a 5 × 5 Latin-Square randomization. In Experiment 6, three separate groups of 20 moths were exposed to 500 μg of codlemone for periods of 10, 20, or 30 min. All pre-exposure treatments ended at the same time. A fourth group of 20 moths, held in a mason jar with solvent only for 30 min, served as the control group. Following the same procedure as above, these four groups of treated and control moths were presented separately at 20-min intervals into the flight tunnel using a 4 × 4 Latin-Square randomization.

Experiment 7: pre-exposure dose × time product

An experiment was conducted to determine whether similar pre-exposure effects could be generated by different treatment regimes giving the same codlemone concentration × pre-exposure time products. Three separate groups of 19–20 moths were pre-exposed to a 5000 μg.min codlemone treatment in mason jars. Moths were held in jars containing 50, 500, and 5000 μg of codlemone, for periods of 100, 10, and 1 min, respectively. All pre-exposure treatments ended at exactly the same time. A fourth group of 20 moths held in mason jars with solvent for 100 min served as the control group. On each of 4 days, these four groups of treated and control moths were presented separately at 20-min intervals into the flight tunnel using a 4 × 4 Latin-Square randomization.

Experiments 8–10: pre-exposure in codlemone-treated moving air

Experiments were conducted to assess the effects of pre-exposing moths to known, varying, amounts of codlemone released into moving air (5–10 cm s^{-1}). Caged moths were hung 20 cm downwind of 3 × 3 cm arrays of nine Celcon-fibre pheromone-sources (Weatherston et al., 1985) in a miniature clear polyester wind tunnel (60 cm long × 15 cm high × 15 cm wide) housed in a fume hood at 23 °C. At 20 °C, each fibre delivers 0.1 μg of codlemone h^{-1} (Knight, 1995). In three separate experiments, groups of 15 moths were given 75-min pre-exposures to arrays of nine pheromone-sources having either 1, 5, or 20 Celcon fibres at each source, respectively, resulting in treatments of 0.9, 4.5, and 18 μg h^{-1} (× 75 min) that are equivalent to 1.1, 5.6, and 22.5 μg.min of codlemone pre-exposure, respectively. Groups of 15 caged moths exposed to clean air in the bioassay flight tunnel for 75 min served as controls. Each pre-exposure treatment and paired control was replicated once in random order on each of 6 days. Rubber septa lures were used in these experiments.

Experiment 11: pre-exposure in codlemone-treated orchard air

To determine if the effects of pre-exposure seen in the laboratory could be duplicated under certain field conditions, caged moths were hung in an apple orchard that had been treated with Isomate-C® commercial pheromone dispensers (Shin-Etsu Chemical Company, Tokyo, Japan). Thirty to 50 caged moths were pre-exposed for 30–34 h in a 1.2 ha Red Delicious apple orchard (3 m tree × 5 m row spacing) that had been treated 1 week earlier with 1000 dispensers ha^{-1}. Dispensers were hung ca. 1 m down from the central leader apices, which on average reached 3 m above ground. Cages were suspended on a wire in the centre of the orchard at the same height as dispensers between two adjacent trees across the tractor alley separating rows of trees. Caged moths hung for the same period in an untreated McIntosh apple orchard located several hundred meters distant from the treated orchard served as control moths. Caged moths were retrieved from orchards 30 min before dusk, chilled in the 2.5 °C cold room under low intensity red lights, marked with fluorescent powders, placed in release cages and transferred to a darkened flight-tunnel room. Both groups of moths were assayed simultaneously, 20 min after acclimating to tunnel conditions which corresponded to the start of their natural scotophase. This entire procedure was repeated over 3 days in August and September, 1996.

At the same time that moths were being pre-exposed in the orchard, 200 male codling moths marked with fluorescent powder were released simultaneously from the centre of each treated and control orchard. Two Pherocon 1-CP style wing traps baited with 1-mg rubber septa lures were hung 20 m either side of the release point. The traps were checked daily and the numbers of moths captured were recorded during the 1-week test period.

Experiment 12: latency of recovery from pheromone exposure

An experiment was conducted to determine how long the behavioural effects of pre-exposure to codlemone would last. Four separate groups of 18–20 moths were given a 10-min pre-exposure to 500 μg of codlemone and assayed 1, 2, 3, or 4 h after the end of pre-exposure. A fifth group of 19–20 moths held for 10 min in an identical mason jar with solvent only served as the control group. In order to introduce groups of moths into the tunnel using a 5×5 Latin-square randomization procedure, it was necessary to readjust the pre-exposure start times for various treatments every day to match the projected flight times. As before, groups of treated and control moths were introduced one at a time into the flight tunnel on each of 5 separate days.

The sensitivity of antennae to codlemone and the latency of recovery after pre-exposure were measured using electroantennograms (EAGs). Our EAG system consisted of an IDAC-02 computer-coupled data acquisition system, an INR-02 EAG-SSR system and AutoSpike software from Syntech (Hilversum, The Netherlands). Recording and indifferent electrodes consisted of silver-coated wire inside glass micropipettes (10 μl microcapillary tubes) containing 0.1 N KCl with 10% polyvinylpyrrolide (v:v). Glass pipettes were pulled at 300 °C on a Narishige (Model PN-30) micropipette puller.

Male insects were 24–48 h old when used for electroantennograms. The protocol followed was to measure an EAG dose–response curve on a naïve male moth while a second moth was being pre-exposed to codlemone in a glass mason jar, after which time a similar dose–response curve was assessed on this pre-exposed moth. EAG measurements alternated between naïve and pre-exposed moths, with a fresh moth set up for pre-exposure and a naïve moth assessed in between. A stimulus sequence of hexane, increasing concentrations of codlemone dissolved in HPLC-grade hexane (3, 10, 30, 100, 300, 1000, and 3000 ng) and hexane again, was applied to excised antennae from individual moths. Each pheromone stimulus was preceded and followed by stimulation with the plant volatile (*E*)-2-hexenal (Sigma Chemical Company, St Louis, MO, USA). Stimulus puffs were generated with a Syntech CS-05 pulse generator and delivered at 10 ml s^{-1}, with 200 ms pulse duration, through glass Pasteur pipettes containing 1×2 cm pieces of Whatman no. 1 filter paper impregnated with varying concentrations of codlemone in 10 μl of hexane. EAGs were measured as the maximum amplitude of depolarization elicited by the applied stimulus. To correct for possible changes in antennal activity over the course of collecting data on an entire dose series, all EAGs were normalized by dividing mV responses to codlemone stimuli by the average mV response measured for (*E*)-2-hexenal (10 μg in 10 μl of paraffin oil) throughout a single antennal recording session. Dose–response EAGs were measured 30–90 min after either 10- or 30-min pre-exposures to 500 μg of codlemone in a glass mason jar and compared to naïve moths exposed to solvent alone.

Statistical analyses

In Experiments 1–3, the frequencies of various pre- and postflight behaviours in moths pre-exposed to 100, 500, and 1000 μg for 30 min and their respective paired controls, were compared using Fisher's Exact Test for 2×2 contingency tables (Zar, 1984). All percentage data were subjected to an arcsine square root transformation before analysis. In Experiments 4, 6, and 7, percentage catches for control and treatment groups of moths were analyzed by an analysis of variance (ANOVA) appropriate for 4×4 Latin-Square experimental designs and mean (n = 4) percentage catches of the pre-exposed treatment groups were compared with mean percentage catches of the control group using Dunnett's test, or to each other using a Student–Newman–Keuls' test (Zar, 1984). In Experiments 8–11a, mean percentage catches of paired treatment and control groups were compared using two-sample t-tests. In Experiment 11b, proportions of marked and released codling moths recaptured in treated and control orchards were compared with a x^2-test. In Experiments 5 and 12, percentage catches were analyzed by an ANOVA appropriate for a 5×5 Latin-square (Zar, 1984) and mean (n = 5) percentage catches of pre-exposure recovery treatments were compared with mean percentage catch of the control group using Dunnett's test. Regression analyses were used to describe the relationships between percentage catch and pre-exposure times (Expts 5 and 6) and the latency of behavioural recovery following pre-exposure to codlemone (Expt 12). All statistical tests were performed with SigmaStat® (Version 3.0, SYSTAT Software Inc., Richmond, CA) and all experimental error rates were set at $\alpha = 0.05$.

Results

Experiments 1–3: behavioural sequence

In single-moth assays, all quiescent, naïve codling moths (control moths) walked while wing fanning and initiated flight in response to introduction to a pheromone plume in the flight tunnel (Figure 1). Percentages of control moths that made the transition from wing fanning to upwind oriented flight and contacting the pheromone lure declined in each experiment (Figure 1), but in almost all cases, naïve moths that locked onto the plume while in upwind flight also completed the behavioural sequence by contacting the source. The average percentage of naïve moths contacting these synthetic pheromone lures across

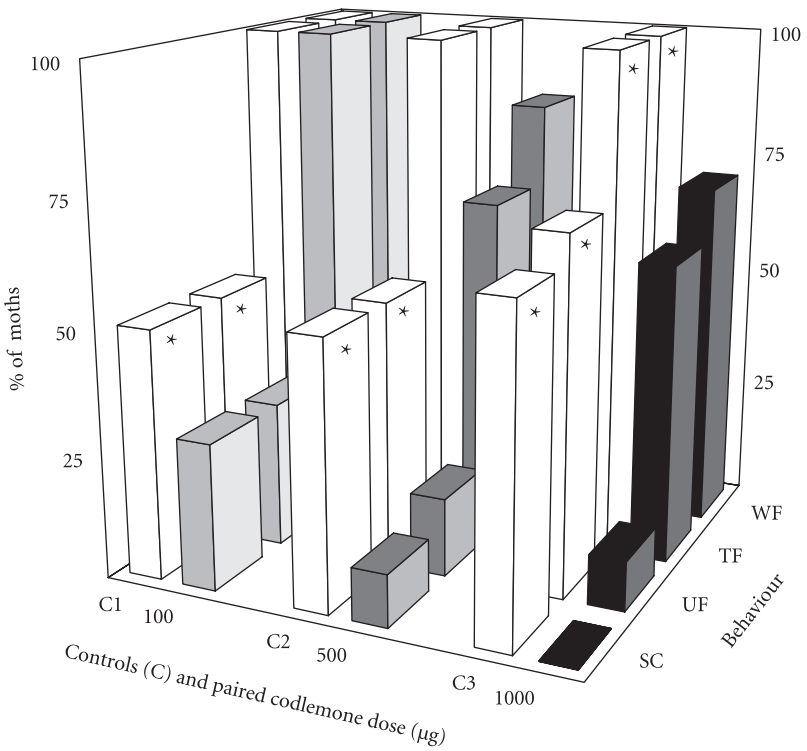

Figure 1 Paired percentages of naïve (control) and pre-exposed male codling moths showing a sequence of pheromone-mediated behaviours (WF = wing fanning, TF = taking flight, UF = upwind flight, SC = source contact) in response to pheromone plumes from 2.5 μg filter paper sources of codlemone in a flight tunnel following 30 min pre-exposure to solvent- or codlemone-treated air of increasing doses delivered from filter paper discs in closed jars. Paired bars within a behavioural category for each pre-exposure dose with an asterisk are significantly different by Fisher's Exact tests ($P \leq 0.05$). Eighteen to 20 moths were observed for each bar.

all experiments (55.9%) was similar to the percentage of naïve moths previously observed (n = 29 males; G.J.R. Judd, M.G.T. Gardiner, N.C. DeLury and G. Karg, unpubl.) contacting a calling female (48.3%) in separate flight tunnel experiments.

Pre-exposure treatment with 100 μg of codlemone for 30 min in a sealed jar had no significant effect on wing fanning and flight activation with respect to control moths (Figure 1). Moths became more quiescent following exposure to 500 and 1000 μg codlemone treatments, as both wing fanning and flight initiation declined with these pre-exposure doses, but were only significantly reduced by the 1000 μg dose (Figure 1). At all treatment doses, significantly fewer pre-exposed moths engaged in upwind flight and contacted the source relative to naïve control moths (Figure 1). Responsiveness at each behavioural step, but particularly source contact (Figure 1) appeared to be dose dependent; source contacts being reduced 42, 79.2, and 100% relative to control moths after pre-exposures of 100, 500, and 1000 μg, respectively. From these collective observations it was concluded that recording source contact, or even near-source contact with these synthetic lures, as would be generated from trap catches, might be as useful for studying the behavioural effects of codlemone pre-exposure as recording the entire behavioural sequence from pre-flight wing fanning to contact. For this reason in all further experiments an assay technique using trap catch as a measurement of behavioural responsiveness was adopted. This trapping assay had an additional advantage in that it allowed the measurement of a greater number of moths, in a shorter period of time, by allowing us to fly groups of moths.

Experiment 4: pre-exposure dose

In group assays, 30-min pre-exposures to 5 and 50 μg of codlemone had no effect on trap catches, but a 30-min exposure to 500 μg significantly reduced the percentage of moths that were caught compared to control groups (Figure 2). The mean percentage of control moths caught in group assays (65.1%) was somewhat higher than the mean percentage of source contacts (55.9%) among all control moths in single-moth assays (Figure 1).

Experiments 5–6: pre-exposure duration

The effects of pre-exposure appeared to occur within a narrow window of time as trap catches declined steeply and significantly relative to the controls with 2–8 min of exposure to the 500 μg source (Figure 3, Expt 5). Experiment 6 revealed very little difference in the effects of pre-exposure to 500 μg of codlemone for durations of 10, 20, or 30 min, but all treatments significantly reduced catches relative to the control group (Figure 3, Expt 6). The relationship between declining mean trap catches and time exposed to the 500 μg source in Expts 5 and 6 collectively, is described accurately ($R^2 = 0.93, P < 0.05$) by a non-linear

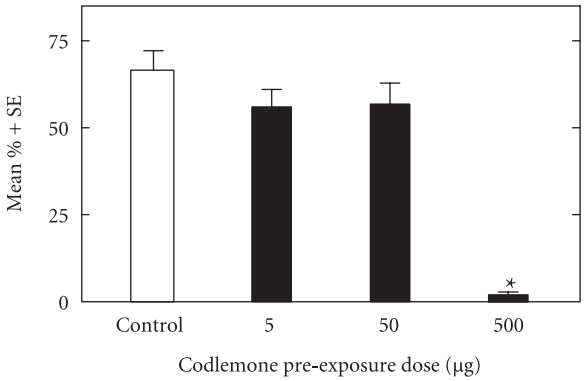

Figure 2 Mean + SE percentages of male codling moths caught in traps baited with 2.5 μg filter paper sources of codlemone in a flight tunnel following 30 min pre-exposure to increasing doses of codlemone or a solvent control. Treatment bars with an asterisk are significantly different from the control group (Dunnett's test, $\alpha = 0.05$) following a significant ANOVA ($P \leq 0.05$). Four groups of 18–20 moths were observed for each bar.

sigmoidal regression model that asymptotically approaches zero:

$$y = 75.6/(1 + \exp^{(-(\text{time}-5.8)/-1.8)}).$$

Over a narrow range of 2–10 min, pre-exposure duration appeared to have a much more pronounced effect on inhibiting subsequent responsiveness to codlemone lures than did pre-exposure dose. Whereas a fivefold increase in pre-exposure time (2–10 min) caused a 69% reduction in trap catches (Figure 3), a 10-fold increase in pre-exposure dose (5–50 μg) caused no increased reduction in trap catches (Figure 2), and only when at a 20-fold dose (100 μg) did a significant effect reveal itself (Figure 1).

Experiment 7: pre-exposure dose × time product

Based on the previous experiments, it was anticipated that pre-exposure dose and time might interact in such a way that the effects of pre-exposure could be predicted from a simple arithmetic product of the two variables. Results from Experiment 7 suggest that the effects of pre-exposure in a closed-jar system did not occur in this way. Although all three pre-exposure treatment regimes provided the same dose × time codlemone exposure product (5000 μg.min l^{-1}) and all caused a significant reduction in trap catches relative to the control group (Figure 4), a 10-min pre-exposure to 500 μg in static air was sufficient to nearly maximize disruption (Figures 3 and 4). Concentrations of codlemone present in this closed system for this latter treatment dose were estimated to be 35.5 ± 1.5 μg l^{-1} (35.5 mg m^{-3}) or a realized exposure of 355 μg.min l^{-1}.

Experiments 8–10: pre-exposure in a wind tunnel

The pre-exposure of moths to all three doses of codlemone delivered in moving air in a miniature wind tunnel had significant effects on trap catches (Figure 5). Disruption relative to controls was dose dependent, increasing from 75 to 99% with a 20-fold increase in pre-exposure dose.

Experiment 11: pre-exposure to codlemone in an orchard

Moths pre-exposed to an Isomate-C® commercial pheromone treatment in an apple orchard for 30–34 h (Expt 11a) were as responsive (50.6 ± 5.8%) to pheromone in the flight tunnel 30 min after being removed from the orchard as

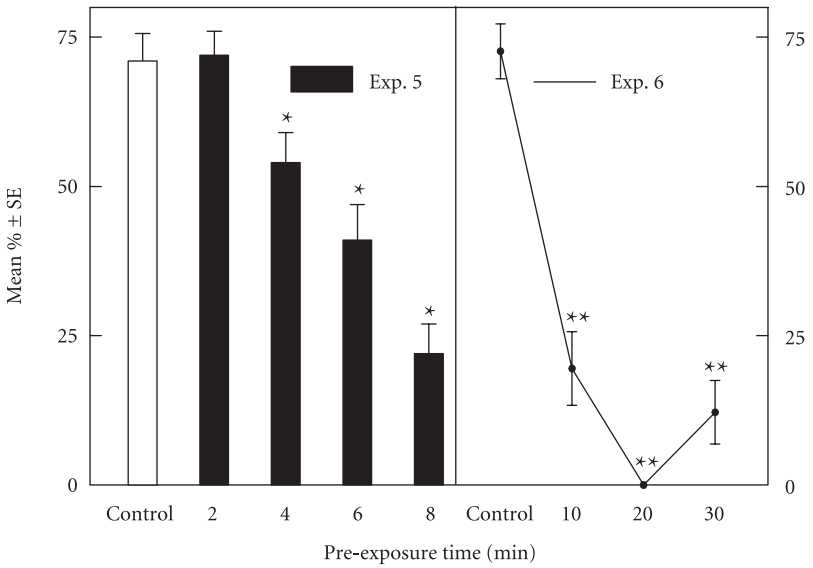

Figure 3 Mean + SE percentages of male codling moths caught in traps baited with 2.5 μg filter paper sources of codlemone in a flight tunnel following pre-exposure to 500 μg sources of codlemone for increasing lengths of time. Treatment bars (Expt 5) or data points (Expt 6) within an experiment having asterisks are significantly different from their respective control group (Dunnett's test, $\alpha = 0.05$) following a significant ANOVA ($P \leq 0.05$). Four groups of 18–20 moths were observed for each bar.

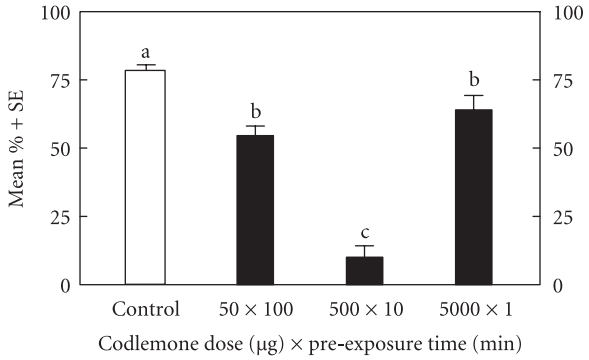

Figure 4 Mean + SE percentages of male codling moths caught in traps baited with 2.5 μg filter paper sources of codlemone in a flight tunnel following different pre-exposure treatment regimes having the same dose × time products of codlemone pre-exposure. Bars with different letter superscripts are significantly different from each other (Student–Neuman–Keuls' test, $\alpha = 0.05$) following a significant ANOVA ($P \leq 0.05$). Four groups of 18–20 moths were observed for each bar.

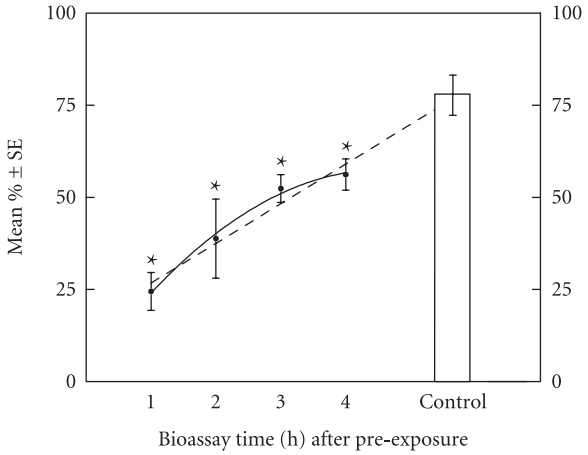

Figure 6 Mean ± SE percentages of male codling moths caught in traps baited with 2.5 μg filter paper sources of codlemone in a flight tunnel at increasing times after 10 min pre-exposure to a 500 μg source of codlemone or solvent control. Treatment data points with an asterisk are significantly different from the control group (Dunnett's test, $\alpha = 0.05$) following a significant ANOVA ($P \leq 0.05$). Five groups of 18–20 moths were observed for each bar. Dotted line is best fit least-squares linear regression of mean recapture vs. time projected through the point of control equivalency and solid line is alternative best fit non-linear regression fitted to the same data without projection to the control data point.

were moths held in an untreated orchard (46.6 ± 7.4). However, the recapture (Expt 11b) of codling moths released at the same time as this exposure was completely inhibited in this Isomate-treated orchard (0%) relative to recapture in the untreated orchard (23%).

Experiment 12: latency of recovery

Moths were slow to recover behavioural responsiveness to 2.5 μg lures following 10-min pre-exposure treatments of 500 μg of codlemone, because 4 h after pre-exposure, the treated moths were still significantly less responsive to pheromone lures than control moths (Figure 6). A linear regression line ($y = 16.01 + 10.76[\text{time}]$) fitted to the mean data ($r^2 = 0.98$, $P < 0.05$) and extrapolated beyond the data set to the point of trap-catch equivalency with catches of control moths (76.7%), predicts that moths may require ca. 5.5 h to recover their full behavioural responsiveness (Figure 6). However, this estimate may be low because behavioural recovery appears to be levelling off 4 h after exposure, and a polynomial regression line ($y = 2.67 + 23.99[\text{time}] - 2.62[\text{time}^2]$) can also describe the data with a slightly better R^2 value (0.99, $P < 0.05$) than the linear model (Figure 6).

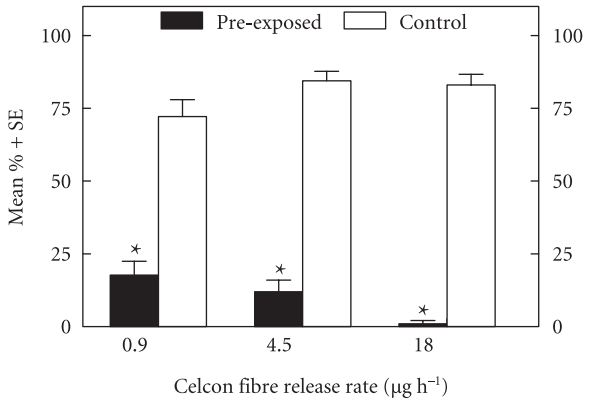

Figure 5 Paired mean + SE percentages of male codling moths caught in traps baited with 10 μg rubber septa codlemone sources in a flight tunnel after 75-min pre-exposures to moving clean air, or increasing doses of codlemone delivered from Celcon® fibres into moving air. Paired bars with an asterisk superscript are significantly different from each other (t-test, $P \leq 0.05$). Six groups of 15 moths were observed for each bar.

Antennae from naïve codling moths showed a fairly typical sigmoid response to stimulation with increasing doses of codlemone (Figure 7). Doses of less than 30 ng produced responses that were indistinguishable from those elicited by hexane, and less than those of the plant volatile standard. EAG responses increased through the mid-range of doses, plateaued and appeared to decline at the highest doses. Excised antennae from moths pre-exposed to 500 μg of codlemone in a jar for 10 min also produced a sigmoid-shaped dose–response curve, but the curve was shifted towards higher doses (Figure 7), suggesting that there is an elevated response threshold. Thirty to 90 min

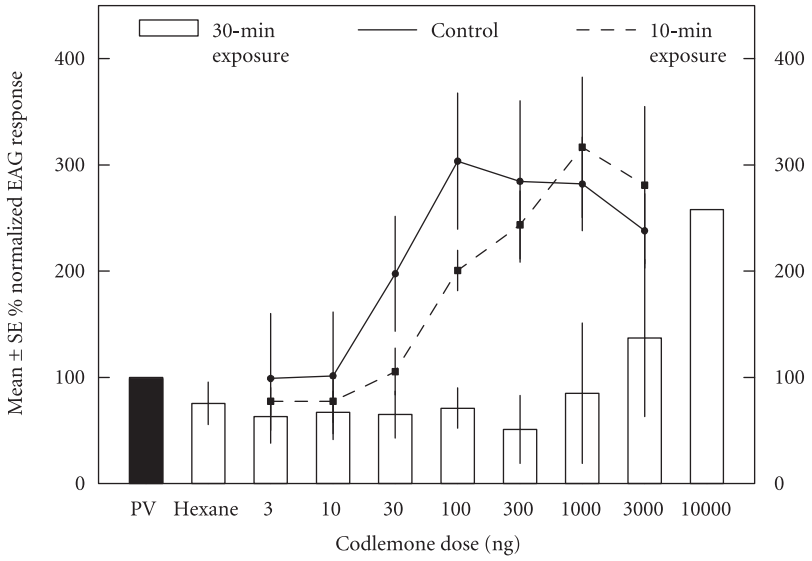

Figure 7 Mean ± SE normalized EAG dose–response relationships generated from excised antennae of male codling moths 30–90 min after 10-min (n = 15), and 30-min (n = 5) pre-exposures to codlemone-treated air delivered from a 500 µg source of codlemone-impregnated filter paper or solvent-treated air controls (n = 15) in closed jars. Each antennal response to a codlemone dose was normalized by expressing mV measured depolarization responses as a percentage of the mean mV response to (*E*)-2-hexenal (PV) for each antennal dose-series.

after exposure, the peak response of these pre-exposed antennae was at 1000 ng, 10-fold greater than naïve antennae. Pre-exposure for 30 min caused an even greater shift in threshold response, as antennae receiving this treatment were 100-fold less responsive to codlemone than naïve moths (compare the responses to 100 and 10 000 ng doses).

Discussion

Our flight tunnel results illustrate a classic behavioural expression of an adaptation-disadaptation effect with pre-exposure to pheromone, but this is not to imply an exclusively peripheral phenomenon. Adapting stimuli of low intensity and short duration had no observable effect on behaviour. When stimulus intensity and exposure times increased, behavioural responsiveness declined linearly and then approached near complete lack of response asymptotically when the dose and/or exposure time was great enough. After removing the adapting stimulus, insects recovered slowly but eventually started to respond both physiologically and behaviourally.

Reduction in response to pheromone lures in our flight-tunnel assays can be explained by at least two physiological mechanisms: (1) sensory adaptation at the antennal neuronal level, and (2) habituation in the central nervous system. Our preliminary data comparing the EAG responses of naïve and pre-exposed codling moths showed that pre-exposed antennae exhibit a form of long-term adaptation that provides a partial explanation for reduced upwind flights, source contacts, and trap catches in response to synthetic pheromone lures in flight tunnel assays. Previously, Kuenen & Baker (1981) documented a short-lived form of antennal adaptation in *Trichoplusia ni* (Hübner) lasting ca. 1 min after removal from pheromone exposure, while Schmitz et al. (1997) characterized adaptation in *Lobesia botrana* (Denis and Schiffermüller) lasting 5–6 min, but more recently Stelinski et al. (2003a) found a form of antennal adaptation lasting at least 12.5 min in *Choristoneura rosaceana* (Harris), that they termed 'long-lasting'. None of these studies however, showed that antennal adaptation was responsible for reduced behavioural responsiveness after pre-exposure, although the adaptation of antennal receptors appeared to influence behaviour during exposure periods (Kuenen & Baker, 1981).

Codling moth appear to exhibit an extreme variant of long-lasting antennal adaptation several times greater than previously reported (Stelinski et al., 2003a), as long as 1.5 h (Figure 7). In codling moth therefore, antennal adaptation must be accounting for at least some of the behavioural effects of pre-exposure up to 1.5 h after exposure. However, because the data suggest that attraction is being suppressed for at least 4 h, well after the antennae have probably recovered (although we did not test this), it seems likely that habituation is involved. If moths are becoming habituated, and this seems reasonable to conclude on the basis of our data (Figure 6), and they are experiencing concurrent antennal adaptation (Figure 7), then our data do not appear to support the hypothesis that antennal adaptation (Bartell & Lawrence, 1977a; Kuenen & Baker, 1981), especially long-term adaptation (Stelinski et al., 2003a), is an impediment to habituation in tortricid moths. If this adaptation-habituation hypothesis is correct, we need an alternative explanation for our data. Perhaps the diene alcohol pheromone used by the codling moth is more readily absorbed by the insects' waxy cuticle under the extreme concentrations presented to them, than is typical of the

acetate and aldehyde pheromones used by Lepidoptera in these other studies. Such a phenomenon may contribute to long-term antennal adaptation or mimic habituation of the central nervous system. A more detailed study of this phenomenon is warranted.

Pulsed pheromone pre-exposure has been shown to cause greater reductions in moth activity, upwind flight, and contact with pheromone sources than constant exposure (Bartell & Lawrence, 1977a,b; Kuenen & Baker, 1981) presumably because the adaptation of receptor neurones is prevented and habituation is promoted. We did not study the effects of pulsed pheromone per se, but we did present pheromone in moving air, which must produce a pulsed pheromone signal relative to the closed jar pre-exposure method, because microfilaments will be created within plumes emitted from individual Celcon fibres. From our laboratory data it appears that pre-exposure to pheromone in moving air (Figure 5) may be more effective at reducing responsiveness than pre-exposure in static air (Figures 1–4) because our calculations suggest that the concentration at which disruption was achieved was much lower in moving air. Considering the highest moving air treatment for example, an 18 μg h^{-1} delivery for 75 min provides 22.5 μg.min of codlemone pre-exposure. A 10-min exposure in a jar treated with 500 μg of codlemone, having an approximate concentration of 35.5 μg l^{-1}, may provide 355 μg.min $l^{-1}$of codlemone pre-exposure. If we take into account the fact that pheromone delivered in moving air is diluted into a much greater volume compared to the jar exposure, then the differences become greater. Accounting for the dimensions and wind speed in our mini-tunnel exposure system, codlemone delivered at 18 μg h^{-1} was diluted into 8100 l of air h^{-1} (= 0.0022 μg l^{-1}), meaning that the insects were potentially exposed to 0.166 μg.min l^{-1} of codlemone in 75 min, or ca. 2100-fold less than the 355 μg.min l^{-1} of codlemone pre-exposure in the closed jar example. Alternatively, the biggest difference in the two presentation procedures could simply be the fact that moving air increases the rate at which pheromone molecules encounter the insects' antennae. However, a 2100-fold difference in dose × time products might be difficult to compensate for. Clearly, the effects of pulsed pre-exposure on both antennal adaptation and habituation in codling moth need further study.

If moving air increases the insects' encounter rate with pheromone, and if the effects of pre-exposure last several hours, then why did moths pre-exposed in orchards (where trap catches were completely inhibited) not show any effects of pre-exposure in subsequent flight-tunnel tests? The emission of codlemone from a 0- to 10-day-old Isomate-C® dispenser under field conditions typical of those in this study is ca. 20 μg h^{-1} at dusk (Knight, 1995). At this emission rate, with 1000 dispensers ha^{-1} and a canopy volume of about 30 000 m^3, maximum codlemone concentrations might be in the order of 20 μg m^{-3}, or 20 ng l^{-1}, and this is only if we assume it is a closed system without wind, pheromone adsorption on plants, and without chemical or photo-degradation of codlemone. During a 30-h field exposure, moths may receive at most 36 μg.min l^{-1} of codlemone exposure, ca. 10-fold less than the 355 μg.min l^{-1} of codlemone exposure needed to cause an effect in the closed-jar tests; but perhaps 200-fold greater than experiments in our miniature wind tunnel suggest (see above). One of the few studies attempting to measure atmospheric pheromone concentrations in orchards resulting from treatment with Isomate dispensers suggests that concentrations are more likely in the ng m^{-3} or pg l^{-1} range (Suckling et al., 1999). Recent air-sampling studies in apple orchards treated with Isomate-C^+ at 1000 dispensers ha^{-1} (V. Hebert and J. Brunner, Washington State University, pers. comm.) confirmed that concentrations of codlemone are in the order of ng m^{-3}, much lower than our results suggest are needed to see any long-lasting adaptation. Previous behavioural studies with *L. botrana* (Schmitz et al., 1997) also concluded that atmospheric concentrations of pheromone resulting from hand-applied dispensers could not cause sensory adaptation because an 8-h exposure in pheromone-treated vineyards had no effect on the subsequent recapture of *L. botrana*. Likewise, Stelinski et al. (2003b) found that the long-lasting antennal adaptation they characterized in *C. rosaceana* (Stelinski et al., 2003a) could only be generated under field conditions when the moths were within centimetres of the Isomate dispensers. Of note here, the estimated concentration these authors suggest causes long-lasting antennal adaptation in *C. rosaceana* was almost identical to the approximate concentrations of codlemone we estimated for our 30-min 500 μg jar treatment.

If average atmospheric concentrations in orchards are not enough to cause long-term adaptation and habituation that could last 30 min, do these neurophysiological effects have a role to play in the disruption caused by these hand-applied dispensers? In tests with Celcon fibres, the upper release rate was chosen because it mimicked release rates for the Isomate-C® dispenser (Knight, 1995) in commercial use when these experiments were done (Judd et al., 1996). In our miniature wind tunnel, moths were partially restrained near these sources, which did not happen in the orchard exposure experiment. If free flying codling moths landed on or near the Isomate-C® dispensers in the field and remained there for several minutes, they could receive an exposure equivalent to that in our wind tunnel system and would probably experience reduced behavioural responsiveness (Figure 5) and perhaps habituation

(Figures 6 and 7). Using field flight tunnels, Cardé et al. (1998) provided evidence that close contact with Isomate 'rope' dispensers leads to habituation of male pink bollworm moths that manifests itself as an elevation of the pheromone response threshold. A similar phenomenon in codling moth may explain why they were not recaptured in our orchard study. In our experience, however, we have never observed a codling moth landing on dispensers in the field, and in a Swedish study, Witzgall et al. (1999) could not find any evidence that male codling moths landed on mating-disruption dispensers releasing codlemone in orchards treated only with codlemone. In untreated orchards however, single disruption dispensers elicited a few landings. The main problem with field observations of this type is that the history of pre-exposure to pheromone is unknown (Witzgall et al., 1999). It is entirely possible that the moths landed within the canopy near dispensers, or were arrested in flight downwind, or were simply attracted into trees with dispensers without ever being visible to observers in these studies.

Even if codling moths show few signs of long-lasting adaptation or habituation after removal from pheromone-treated orchards, it does not mean that these mechanisms are not acting while moths are in the orchard. Codling moths are known to be more responsive to higher-load codlemone lures (10 mg loaded septa) in Isomate-C®-treated orchards compared with untreated orchards (Judd et al., 1996) which may be an indication of partial neuronal adaptation. Mafra-Neto & Baker (1996) showed that pre-exposure to pheromone can elevate threshold responses for pheromone-mediated behaviours, and our EAGs suggest this may also happen in the codling moth (Figure 7). However, response to high-load lures in backgrounds of pheromone could also be indicating that camouflage of plume boundaries from low-load lures is occurring, and therein lies the difficulty of interpreting field experiments.

In terms of improving mating disruption of codling moth by invoking more mechanisms, perhaps as others have suggested (Minks & Cardé, 1988; Cardé & Minks, 1995; Witzgall et al., 1999), more complete pheromone blends and blends lacking behavioural antagonists are necessary to maximize false-trail following, induce more landings, or at least nearby approach, to the point where neurological effects can manifest themselves. Our data suggest that if codling moths were near high-release-rate pheromone sources for long enough, then long-lasting adaptation and/or habituation could be important in reducing subsequent response to females. However, unique delivery systems designed to do this (Shorey & Gerber, 1996) have not yet lived up to early expectations for codling moth control in North America (G.J.R. Judd and M.G.T. Gardiner, unpubl.). A different approach is perhaps needed to increase the potential for mechanisms like long-term adaptation and habituation to act. Clearly, more work is needed to develop delivery systems and formulations that can maximize longer term neurological effects (Kuenen & Baker, 1981), because as Cardé et al. (1998) pointed out, successful disruption of communication will likely involve a complex interplay of mechanisms. Detailed laboratory and field examination of the mechanisms of communication disruption may provide an understanding of how best to achieve this interplay and allow us to develop more effective mating-disruption systems.

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