

Identification of *Cameraria ohridella* sex pheromone and its possible use in horse chestnut protection

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Abstract: The major component of sex attractant released by the virgin female of the horse-chestnut leafminer *Cameraria ohridella* Deschka et Dimiæ (Lepidoptera: Gracillariidae), which devastates horse chestnut trees in Europe, was identified in picogram quantities as (8E,10Z)-tetradeca-8,10-dienal. The spectral methods were not used, the identification relied entirely on alternative analytical methods like 1) gas chromatography with electroantennographic detection (GC-EAD), 2) calculation of Kovats' indices of the active principle on different GC phases, and 3) construction of EAG response profiles to C12 and C14 saturated and unsaturated standards with different functional groups. The synthetic pheromone was prepared by a stereospecific synthesis and shown to be highly active for conspecific males and was proved to be fully comparable to the natural substance in all respects. The potential use of the pheromone to protect horse chestnut trees in Europe is discussed.

Key words: sex pheromone, identification, horse-chestnut leafminer, *Cameraria ohridella*, Lepidoptera, Gracillariidae, EAG, GC-EAD, wind tunnel, field test, delta traps

Introduction

The horse-chestnut leafminer *Cameraria ohridella* Deschka & Dimiæ (Lepidoptera: Gracillariidae) is presently the most dangerous pest of horse-chestnut, *Aesculus hippocastanum* L., in Southern and Central Europe. This pest gradually radiated from the original place of occurrence in Macedonia (Deschka & Dimiæ 1986) to Austria, Hungary, Germany, Slovakia and the Czech Republic (Skuhřavy 1999). *C. ohridella* can have up to four generations a year and the infested trees are usually completely defoliated before the end of the season. When trees are precociously defoliated for several consecutive years they can eventually die, which greatly affects the environment in urban areas.

Current literature reports bionomical data of the species, until now chemical communication has not been studied. At present, the possibilities to control this pest are rather limited (raking of the damaged leaves, spraying with insecticides). The identified sex pheromone can represent a basis for an alternative - designing an integrated pest management (IPM) program.

Materials and methods

Insects

Insects were collected either as adults from naturally occurring populations in Prague (park Stromovka and Royal Garden of Prague Castle) in the morning hours from 22 July to 28

September, 1998 or infested chestnut leaflets were taken to the laboratory, immersed in water and kept in fine fabric-netted cages (0.4 x 0.4 x 0.4 m) at room temperature. Alternatively insect emerged from pupae overwintering in collected dried fallen leaves outdoor. Emerged adults were periodically removed to prevent mating. Males and females were kept separately in glass containers with perforated polyethylene stoppers at LD 14:10 and 24 °C. Females were observed during photophase to determine their calling period. Males were maintained at 5 °C until used in behavioural and electrophysiological (EAG) experiments.

Sample Preparation and Extraction

Calling virgin females (1 to 2 days old) were cooled to –20 °C for 5 min. The abdomen of a female was squeezed with a pair of forceps under a binocular microscope and the extruded abdominal tip was excised between the 7th and 8th abdominal segments, extracted with hexane (ca 10 µl per abdominal tip) and the solution was stored at –20 °C.

Chemicals

High purity grade hexane (Fluka) was used for extractions of calling females and a sample preparation for GC analyses. Series of dodecen-1-yl, tetradecen-1-yl acetate, dodecen-1-ol and tetradecen-1-ol monoenes were obtained from IPO-DLO (Wageningen). Dodecenals and tetradecenals series were prepared from the corresponding alcohols with PCC oxidation (Svatos *et al.* 1999). The geometric isomers of the 7,9-; 8,10-; and 9,11-tetradecadienals were prepared in our laboratory (Svatos *et al.* 1999, Hoskovec & Svatos 2000).

EAG Recordings

A male to be used in EAG recordings (Hoskovec *et al.* 1996) was placed in the tip of a disposable pipette (200 µl, Eppendorf) with a cut end. The exposed head and antennae were fixed in place by melted wax. Electrical activity from the fixed antenna was recorded by using glass Ag/AgCl microelectrodes filled with insect haemolymph saline. Dissected female glands or extracts were applied to a filter paper disc (10 mm) inserted into a Pasteur pipette. Stimuli were delivered onto the prepared antennae by air pulses blown through the pipette.

Wind tunnel bioassay

C. ohridella males (3–4 days old) were flown in a wind-tunnel (Hoskovec *et al.* 1996). Air velocity was maintained at 0.4 m/s. The experiments were performed from 2 to 3 h after the beginning of photophase. Males were released from the middle part of the tunnel into an odour plume which was created by pinning a filter paper disc (7 mm, Whatman No. 2) loaded with the test stimulus onto a holder placed centrally near the upwind end.

Field tests

The field tests were performed in the upper part of the Royal Garden of Prague Castle from 22 July to 28 September, 1998 and from 1 May to 10 September 1999 in park Stromovka (Prague). Delta traps (25 x 10 cm), baited either with three virgin females or with three males housed in small metallic cages (2 x 1.5 cm) and fitted with sticky inserts (Tanglefoot glue) were suspended in the lower part of the horse-chestnut canopy ca 2.5–3 m above ground level. The traps were checked daily and trapped males were identified and counted. Cages without lure were used as control.

Results

Traps with virgin females caught a substantial number of conspecific males when compared to the control traps (Table 1). Traps baited with males were not attractive. The insects caught in control traps were either different microlepidopteran species or mixtures of *C. ohridella* males and females. When a series of 12:Ac and 14:Ac monoenes and their mixtures were tested in the field, none of those chemicals selectively attracted *C. ohridella* males, nevertheless, several different related insect species were trapped (Svatos et al. 1999a). Interestingly, some of the tested compounds showed notable EAG potentials (Z10-14:OAc >>E6-12:OAc).

Table 1. Catches of *C. ohridella* males in Delta traps, Royal garden of the Prague castle, 1998.

Lure:	3 Females	3 Males	Control
Date	Moths caught: <i>C. ohridella</i> males (other species)		
30.7. ¹	113 (27)	nd	5 (1)
10.9. - 12.9. ²	56 (14)	1(0)	1 (0)
21.9. - 28.9. ²	18 (3)	0 (0)	0 (0)

nd: not determined, ¹ third generation, ² fourth generation.

When virgin females were observed at the beginning of photophase, more than 80% showed a typical calling position as was described by Mozuraitis et al. (1997). Females were calling from the first post-emergence day for ca 3 h, with a maximum frequency at 1.5 h of photophase period. Females ceased calling after the 4th day of their life. One dissected female abdominal tip, presumably containing the pheromone gland, was highly attractive for *C. ohridella* males in wind tunnel experiments. Large numbers (90%) of tested males took off and flew in the odour plume, 20% reached the odour source and made copulatory attempts (N = 20). When a similar preparation (from three females) was inserted into a glass Pasteur pipette and blown over male antennae, significantly higher EAG potentials (0.28 ± 0.04 mV, N = 55) were measured than for the control preparation (0.02 ± 0.05 mV, N = 55). Hexane extracts of three female abdominal tips loaded on a paper disc was very attractive for *C. ohridella* males in the wind tunnel bioassay, and the attractiveness was comparable to that elicited with the abdominal tip preparation. The extract (from one tip) elicited an EAG response comparable to excised abdominal tips.

The GC-EAD (male antenna was used as a biological detector, Struble & Arn 1984) examinations of pheromone gland extracts showed pronounced antennal activity on the EAD trace, but no corresponding GC peak was detected by FID detector (Figure 1A). Furthermore, when ~ 100 female equivalents (FE) were injected on a GC/MS (ion trap) instrument no reliable mass spectrum was obtained from the EAD-active area. Clearly, the only analytical tools available were: 1) retention behavior of the EAD active peak on different GC phases, 2) an examination of antennal specificity to libraries of pheromone-like synthetic compounds (EAG response profiles), and 3) micro-derivatizations of gland extracts combined with EAG. Kovats' indices (KIs) of the EAD peak (Figure 1A) were determined using several GC phases of increasing polarity and the measured values were compared with KIs of straight-chain aldehydes, alcohols and acetates (Table 2).

Table 2. A comparison of Kovats' indices^a of the EAD active peak in *C. ohridella* female extracts with some synthetic compounds

GC phase ^b	Female extract ^c	Compounds			
		12:Ac	14:Ald	14:OH	8E,10Z-14:Al ^c
DB-1 ^d	1623.3	-	-	-	1623.9
DB-5 ^d	1674.4	1605.9	1610.8	1675.4	1674.3
DB-WAX ^e	2031.2	-	-	-	2031.8

^a based on saturated hydrocarbons; ^b J & W Scientific, 30m _ 0.25 mm, film thickness 0.25 mm; ^c for the EAD trace; ^d 170 °C; ^e 140 - 240 °C @ 5 °C / min

Based on these measurements, a series of all geometric isomers of dodecen-1-yl acetates, tetradecen-1-ols and tetradecenals and their saturated congener (1 µg) were tested on the EAG preparation. Both KIs and the EAG profiles obtained clearly showed that the pheromone should bear the aldehyde functionality and that unsaturation must be situated near the C-9 atom. The aldehydic nature of the pheromone was confirmed by micro-derivatization experiments where the hexane extracts, treated with *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride in methanol solution (Svatos *et al.* 1999b) were tested both on EAG and in a wind tunnel. The pheromonal activity diminished after this derivatization. However, (*E*)-tetradec-9-enal which had the highest EAG potential (from EAG profiles) showed low behavioral activity (wind tunnel bioassay) and a different KI (1601.8 on DB-5) in comparison to the natural extract. Therefore, it was not considered to be the sex pheromone.

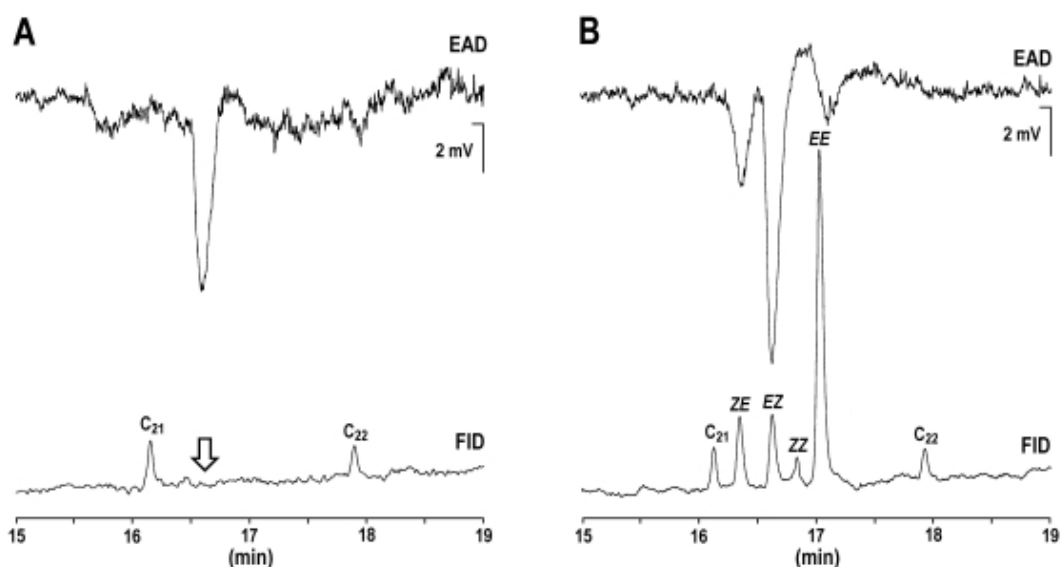


Figure 1 Sections of GC-FID-EAD traces; **A**: a hexane extract of *C. ohridella* calling females (~ 5 FE); **B**: a synthetic mixture of 8,10-tetradecadienal (7) isomers (100 ng) on DB-WAX phase, both co-injected with hydrocarbon standards (C₂₁, 5 ng).

From comparison of KIs on DB-1 and DB-5 phases it seems reasonable to speculate that the pheromone should have more double bonds. Its KI on the DB-5 phase is much higher than the respective KI on a DB-1 phase, which usually points towards conjugation (Attygalle & Morgan 1988). Based on this consideration, and on the measured EAG profiles we prepared mixtures of all geometric isomers of 7,9-, 8,10-, and 9,11-tetradecadienals (Svatos et al. 1999b).

The EAG examination of the mixtures of geometric isomers showed that only an isomeric mixture of 8,10-tetradecadienal isomers (8,10-14:Al) displays the highest antennal activity. Geometric isomers of the 8,10-14:Al were reasonably separated on GC capillary columns and we were able to obtain GC-EAD of the individual isomers (Svatos et al. 1999b). Although males' antennae were, to some extent, sensitive to more than one geometric isomer in the mixture we could clearly eliminate the Z8,E10-14:Al and E8,E10-14:Al isomers. The E8,Z10-14:Al isomer showed higher EAD activity than Z8,Z10-14:Al isomer (Figure 1B). When E8,Z10-14:Al was measured on GC-EAD using several GC phases the corresponding EAG activity showed identical retention behavior (at sub-ng amounts) to hexane-extracted female abdomens (Table 2).

In wind tunnel behavior assay 1 - 0.1 pg of the E8,Z10-14:Al isomer displayed high attractiveness, which was comparable to 3 FE of gland extract (Figure 2). In contrast, the pure Z8,Z10-14:Al displayed a different KI to the natural extract and its behavioral activity was ne-

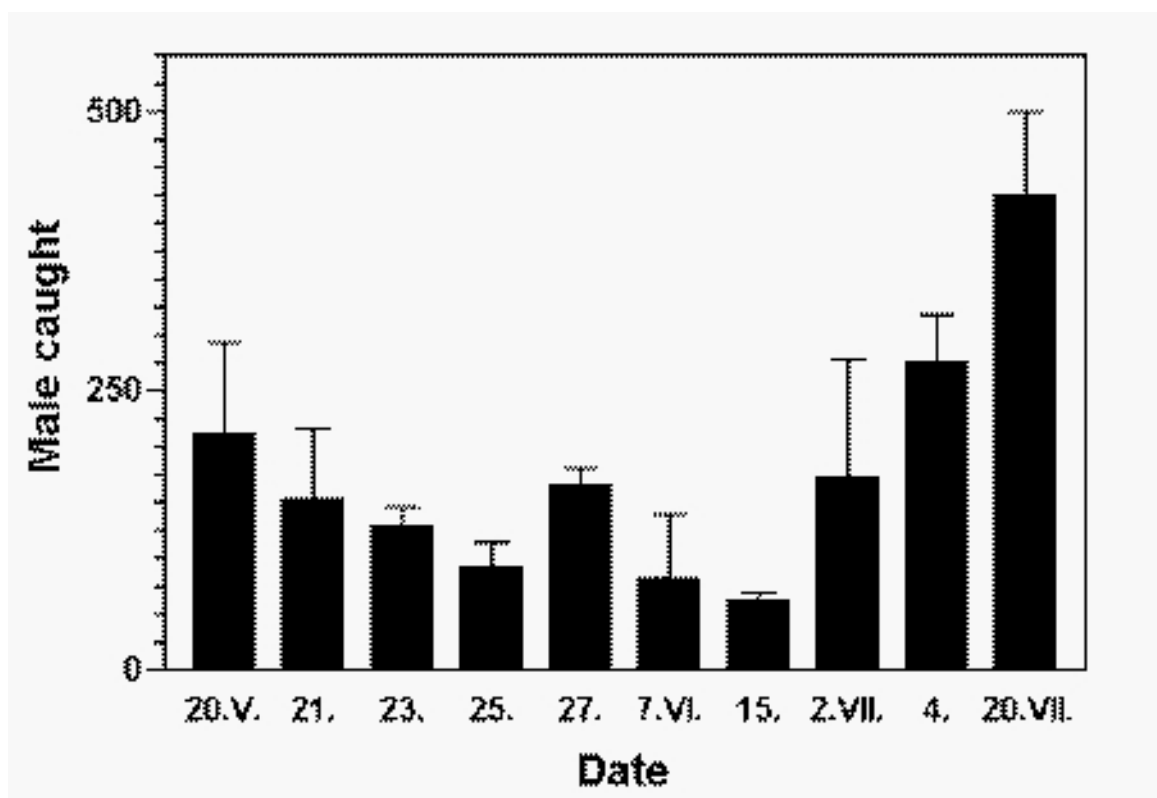


Figure 2 Catches of σ -traps baited with synthetic sex pheromone (sticky inserts, rubber septum, N = 3). Each column represents number of *C. ohridella* males caught per day and trap. The bars represent S.E.

glibile. In preliminary field experiments sticky traps baited with 5 ng of E8,Z10-14:Al isomer (loaded on BBL Taxo paper disc, 1/2 inch dia) were, similarly attractive for *C. ohridella* males as virgin females. All the presented data confirm that E8,Z10-14:Al isomer is the main component of *C. ohridella* sex pheromone.

First field experiments with Delta-traps baited with a rubber septa lured with synthetic pheromone showed high attractiveness and selectivity of the pheromone. Preliminary results are presented in Figure 2.

Discussion

So far sex pheromones were identified for three *Phyllonorycter* species, which are related to *Cameraria*. Females of the tentiform leafminer, *P. mespilella* Hübner, produce E10-12:OAc accompanied by trace amounts of E4E10-12:OAc (Gries *et al.* 1993). For the tentiform leafminer moth, *P. ulmifoliella* Hübner, Z10-14:OAc was identified as the sex pheromone (Mozuraitis *et al.* 1997). Similarly for the apple leafminer, *P. ringoniella* Matsumura, where the pheromone is a mixture of E4Z10-14:OAc and Z10-14:OAc in the 6/4 ratio (Boo & Jung 1996). Structural similarities of sex pheromones in related species lead us to the assumption that *C. ohridella* can, in part, employ similar chemicals. But field tests showed that none of the tested compounds (isomeric dodecenols, tetradecenols and the corresponding acetates) is selectively attractive for *C. ohridella* males (Svatos *et al.* 1999a). Similar results were independently obtained by other research groups (G. Szöcs personal communication). Based on those results reported attempts of several commercial firms to use dispensers attractive for *Phyllonorycter* spp. for monitoring of *C. ohridella* seem questionable.

(8E,10Z)-Tetradeca-8,10-dienal is, to the best of our knowledge, a new sex pheromone and the first identified sex pheromone in the genus *Cameraria*. Other isomers of 8,10-14:Al have been described as attractants for males of other Lepidopteran species; 8E10E-14:Al for *Acrocercops* sp, (Ando *et al.* 1980) and 8Z10Z-14:Al for *Phyllonorycter* sp. (Reed & Chisholm 1985). The E,Z conjugated double bond system is quite common, found for example in bombykol, the first identified sex pheromone.

The high activity of 8E10Z-14:Al would be advantageous for establishing of an IPM system of horse chestnut trees protection. Determination of the onset of the miner's first generation will be helpful to determine proper timing for using of selective insecticides and additionally in forecasting of damages of foliage at the end of season. The second possibility will be to adopt male confusion technique (Cardé & Minks 1995). This control method has been proved to be successful e.g. in orchards (*C. pomonella*) and vineyards (*L. botrana*), the usability in the case of isolated horse chestnut trees must be proved. The third method can be based on using pheromone-baited traps contaminated with insecticide (trap-and-kill) or insect growth regulators.

The high selectivity and attractiveness of the pheromone will possibly allow us to find the original place of *C. ohridella* occurrence and to study mechanism which keeps its population low there. In the case that a larval parasitization, entomopathogens, viruses and similar biotic factors will be localized we shall try to introduce them to Europe as an agent for biological control of the pest.

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