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PHEROMONE VARIATION AMONG EASTERN EUROPEAN AND A WESTERN ASIAN POPULATION OF THE TURNIP MOTH *Agrotis segetum*[1]

BILL S. HANSSON,[2] MIKLÓS TÓTH,[3] CHRISTER LÖFSTEDT,[2] GÁBOR SZÖCS,[3] MITKO SUBCHEV,[4] and JAN LÖFQVIST[2]

[2]*Department of Animal Ecology*
Lund University
Helgonav. 5, S-223 62 Lund, Sweden

[3]*Research Institute for Plant Protection of the Hungarian Academy of Sciences*
Pf 102, H-1525 Budapest, Hungary

[4]*Bulgarian Academy of Science*
Institute of Zoology
Blvd. Russki 1, 1000 Sofia, Bulgaria

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Abstract—The female sex pheromone composition and the male electrophysiological response with respect to the three main sex pheromone components, (Z)-5-decenyl acetate, (Z)-7-dodecenyl acetate, and (Z)-9-tetradecenyl acetate, were investigated in populations of *Agrotis segetum* from Armenia and Bulgaria. The percentage composition of the female-produced pheromone was 1:52:47 and 1:42:57 for the respective populations. Corresponding male receptor frequencies were 9:90:1 and 6:92:2. EAG response profiles of the male antennae were similar for the two populations. The populations from Armenia and Bulgaria differed from the earlier investigated French and Swedish populations, which have larger amounts of (Z)-5-decenyl acetate in gland extracts and have a majority of (Z)-5-decenyl acetate-sensitive receptors. Investigation of receptor frequencies on antennae of male Hungarian moths showed that individuals could be classified as either Swedish or Armenian/Bulgarian type. Males of the Swedish type were preferentially attracted to the three-component pheromone blend, whereas blends of (Z)-7-dodecenyl and (Z)-9-tetradecenyl acetate, and (Z)-7-dodecenol [pure

[1] Schiff. (Lepidoptera: Noctuidae).

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or in mixture with (Z)-5-decenol] attracted the Armenian/Bulgarian type. The nature of pheromone variation among European and Asian populations of the turnip moth and possible mechanisms maintaining the variation are discussed.

Key Words—Sex pheromone, *Agrotis segetum*, Lepidoptera, Noctuidae, (Z)-5-decenyl acetate, (Z)-7-dodecenyl acetate, (Z)-9-tetradecenyl acetate, population variation, olfactory receptors, single sensillum response, gas chromatography, field trapping.

INTRODUCTION

Moth sex pheromones are species-specific communication systems for mate location. Theoretically the sex communication system in a species should be under strong stabilizing selection as long as the species inhabits a continuous area with similar selection pressures on the communication system throughout, due to the great reduction in mating success that an aberrant individual would experience. However, significant intraspecific variation in the pheromone composition has been documented in, for instance, the larch budmoth *Zeiraphera diniana* (Guerin et al., 1984; Priesner, 1979), the European corn borer *Ostrinia nubilalis* (Anglade et al., 1984), and the turnip moth *Agrotis segetum* (Arn et al., 1983, Löfstedt et al., 1986).

The sex pheromone communication system of *Agrotis segetum* has been studied extensively during the last decade. The main pheromone components have been identified as (Z)-5-decenyl, (Z)-7-dodecenyl, and (Z)-9-tetradecenyl acetate (Z5–10 : OAc, Z7–12 : OAc, and Z9–14 : OAc) (Bestmann et al., 1978; Arn et al., 1980; Tóth et al., 1980; Löfstedt et al., 1982). These three substances have also been shown to be detected by olfactory receptor cells situated in three physiologically distinct types of sensilla (Löfstedt et al., 1982; Van Der Pers and Löfstedt, 1986; Löfstedt et al., 1986).

Studies on a Swedish population of turnip moths showed a female pheromone production of Z5–10 : OAc, Z7–12 : OAc, and Z9–14 : OAc in a 4 : 52 : 44 ratio and male pheromone-sensitive sensilla responding to the three components in the ratio 66 : 33 : 1 (Löfstedt et al., 1986). When the Swedish population was compared to a French and a British, the French population was significantly different showing a 47 : 40 : 13 relationship between the produced pheromone components and a 87 : 12 : 1 ratio between the sensillum types. The British insects did not differ significantly from the Swedish (Löfstedt et al., 1986). French males could, in field tests, be attracted to pure Z5–10 : OAc (Arn et al., 1983), which was impossible with the Swedish insects (Löfstedt and Löfqvist, unpublished). Analysis of female pheromone production as well as analysis of male electrophysiological and behavioral response to pheromone components

has thus far demonstrated the existence of at least two different pheromone types among European turnip moths, hereafter referred to as the Swedish and the French types.

In field-trapping experiments performed in Bulgaria the differences between European populations of the turnip moth were demonstrated once more by Subchev et al. (1986), who showed that in this area male turnip moths were attracted to pure Z7–12 : OAc or Z9–14 : OAc. These two substances, mixed at a 1 : 4 ratio, also provided the best bait for the Bulgarian population.

The differences in sex pheromone composition among different geographical populations of *A. segetum* discovered in these studies suggested the present investigation to test the hypothesis that the relative amount of Z7–12 : OAc and Z9–14 : OAc in the female sex pheromone and the number of Z7–12 : OAc and Z9–14 : OAc sensitive sensilla on the male antenna are larger in individuals originating from populations from the east of Europe, compared to individuals from populations of more western or northern origin. Turnip moths from Hungary, Bulgaria, and the Soviet Republic of Armenia were collected, and the female sex phereomone composition and the ratio of different physiological sensillum types on the male antenna were determined.

METHODS AND MATERIALS

Insects. Larvae of *A. segetum* were collected from alfalfa fields near Yerevan, Armenia. Bulgarian and Hungarian moths were supplied from cultures established from eggs of feral females caught by light traps. The larvae were reared on a semisynthetic diet (Nagy, 1970) at 28°C and an 18-hr/6-hr light–dark cycle. The pupae were sexed, and the males were shipped to the Swedish laboratory for electrophysiological single sensillum recordings or their electroantennographic response profiles were investigated in the Hungarian laboratory. Males used for electrophysiological experiments were from the second and third laboratory generations. The females were allowed to emerge in Hungary. Two days after emergence, the female pheromone gland was excised and extracted in hexane, whereafter the extracts were sent to Sweden for analysis. The females extracted were from the first, second, and fourth laboratory generations. Feral males used in the study were caught in pheromone traps in Hungary (see Field Experiments) and were transported live to Sweden to be examined electrophysiologically using the single-sensillum recording technique.

Field Experiments. Hungarian *A. segetum* males for electrophysiological investigation, caught on different baits, were collected from sticky traps in Hungary.

Field tests were conducted using triangular traps made from transparent

polyethylene sheets. Baits were applied in 10 μl hexane solution to 1-cm pieces of red rubber tubing (Bora'szati Szaküzlet, Budapest). All chemicals were supplied by Dr. S. Voerman (Institute of Pesticide Research, Wageningen, The Netherlands). The trapping experiments were conducted May 27–28 and August 1–2, 1988, in Budakeszi, Pest County, Hungary.

In a first experiment the traps were baited with 500 μg Z7-12:OH. In a second experiment the traps were baited with five different lures. The first series was baited with Z5-10:OAc, Z7-12:OAc, and Z9-14:OAc in a 1:1:1 mix; the second series was baited with Z7-12:OAc and Z9-14:OAc in a 1:1 mix; the third series was baited with Z5-10:OAc and Z7-12:OAc in a 1:1 mix. In these three series, the amount of each component was 20 μg. The fourth series was baited with Z7-12:OAc only in a very low amount (0.05 μg), and the fifth series was baited with Z5-10:OH and Z7-12:OH in a 1:1 mix in very high amounts (500 μg of each compound). The last two series were included as they had been shown to attract large numbers of males in earlier field trapping experiments in Hungary (Tóth and Szöcs, personal communication). Insects caught in the traps were gently removed from the traps, put into envelopes, and transported to Sweden, where they were investigated electrophysiologically one to two days later.

Chemical Analysis. Extracts of female pheromone glands were analyzed by gas chromatography on a HP 5880 gas chromatograph equipped with a flame-ionization detector. The sealed ampoules were opened and 6 ng of an internal standard [(Z)-8-tridecenyl acetate] in 3 μl hexane was immediately added. After concentration of the sample to approximately 5 μl at room temperature, the hexane extract was injected on a 30-m-long × 0.25-mm-ID fused silica DB-wax column (cross-linked polyethylene glycol) (J&W Scientific, Folsom, California 95630). Conditions of chromatography were: hydrogen carrier gas velocity 40 cm/sec at 80°C; injector temperature 225°C; split valve opened 1 min after injection; temperature maintained at 80°C for 2 min following injection and then programmed at 10°C/min to 230°C. The amounts of the pheromone components were calculated relative to the internal standard comparing peak heights.

The precision of the quantification was estimated by the analysis of a synthetic reference mixture on five occasions during the two days of insect analysis. Approximately 5 μl of a hexane mixture containing 0.3 ng of the internal standard per microliter and five pheromone gland constituents (see Results) were injected each time.

Electrophysiological Methods. The response profiles of Armenian and Bulgarian male *A. segetum* antennae were recorded using the electroantennographic technique (EAG) (Schneider, 1957). Whole-body preparations were used. One platinum electrode, connected to ground, was inserted into the abdomen of the insect, and one, connected to a high impedance amplifier, was inserted into the

tip end of the antenna. The responses were measured in millivolts, and, to correct for antennal fatigue, the response to a reference substance (Z5–12:OAc) was measured between stimulations with the test compounds. One microgram of a compound to be tested was applied on a piece of filter paper, which was placed in a Pasteur pipet. A puff was then distributed from the pipet into an airstream flushing over the preparation.

Single sensillum recordings were performed with the tip-recording technique (Kaissling, 1974; Van Der Pers and Den Otter, 1978). An antenna was excised from a male moth and placed in a pipet electrode filled with Beadle-Ephrussi Ringer. The electrode was connected to ground by an Ag–AgCl wire. A pheromone-sensitive olfactory sensillum trichodeum was cut using two microscopic glass knives and a second pipet electrode, with an opening of about 2 μm, was positioned over the cut surface of the sensillum. This electrode was connected to a high-impedance amplifier by an Ag–AgCl wire. The stimulus was dispensed on filter paper. One microgram of the Z5–10:OAc and 10 μg each of the other behaviorally active pheromone components (Z7–12:OAc and Z9–14:OAc) were tested. The stimulus paper was put into a 5-ml plastic disposable syringe. One milliliter of the syringe atmosphere containing the stimulus molecules was then injected into a purified and humidified airstream flushing over the antennal preparation at a speed of 0.5 m/sec. The receptor potential and the action potentials elicited in the sensillum by the stimulus were visualized on a digital storage oscilloscope and recorded on tape on a RACAL four-channel tape recorder.

To determine the relative frequency of receptor cells for an individual male, a sample of 10 sensilla, chosen randomly on the third, fourth, or fifth segment from the base of the male antenna, was examined.

RESULTS

Chemical Analysis. Analysis of the synthetic reference mixture ($N = 5$) demonstrated the precision of the gas chromatographic quantifications. The major pheromone component Z7–12:OAc amounted to 2.58 ng (coefficient of variation 2.3%). The relative amounts of the other compounds (Z7–12:OAc always 100 by definition) were: Z5–10:OAc 17.8 (coefficient of variance 1.8%); Z7–12:OH 23.8 (1.5%); Z9–14:OAc 35.2 (3.7%); Z11–16:OAc 2.4 (10.8%).

Compounds in the pheromone gland extracts (Table 1) were identified by comparing their retention times to those of synthetic standards. Based on the batch extracts ($N = 6$, each batch containing extracts from about 10 females) the Z7–12:OAc titer of an average female *A. segetum* was 0.17 ng (range 0.04–0.33) and 0.016 ng (range 0.002–0.042) for the Armenian and Bulgarian insects, respectively. The low titer of the major pheromone component did not allow

TABLE 1. RELATIVE AMOUNTS OF ACETATES AND ALCOHOL (AVERAGE AND RANGE) IN GLAND EXTRACTS FROM TWO POPULATIONS OF *Agrotis segetum*[a]

Population	Z5-10:OAc	Z7-12:OAc	Z7-12:OH	Z9-14:OAc	Z11-16:OAc
Armenian	<2.5	100	88 (65-117)	90 (69-11)	35 (25-51)
	(*N* = 3)	(*N* = 6)	(*N* = 6)	(*N* = 6)	(*N* = 6)
Bulgarian	<2.4	100	179 (51-316)	134 (85-221)	68 (34-138)
	(*N* = 2)	(*N* = 6)	(*N* = 6)	(*N* = 4)	(*N* = 4)

[a]Z7-12:OAc = 100 by definition.

precise quantification of the minor components, nor was positive identification possible for all compounds earlier reported (Löfstedt et al., 1986). The behaviorally active acetate Z9-14:OAc was abundant in both the Armenian and Bulgarian insects, whereas the relative amount of Z5-10:OAc was below 3% of the Z7-12:OAc amount in both populations. The limit of quantification was set by the baseline noise level and the titer of the major pheromone component. In those extracts of the Armenian and Bulgarian populations that contained the largest amounts of Z7-12:OAc, the relative Z5-10:OAc titers were 2.4 and 0.7 (or less), respectively. Thus the percentage composition of the pheromone gland secretions from Armenian and Bulgarian females was calculated as 1:52:47 and 1:42:57, respectively, with regard to the homologous acetates Z5-10:OAc, Z7-12:OAc, Z9-14:OAc. It should be emphasized that the Z5-10:OAc figures are maximal estimates and that the real figures might be even lower.

Electrophysiological Analysis. Of about 100 single sensilla investigated in each population, the Armenian males possessed 9% Z5-10:OAc specialized sensilla and the Bulgarian males 6% compared with the 66% of the Swedish insects and 87% of the French (Figure 1). Thus there is a significant difference in the proportion of the different sensillum types on the male antenna among the Armenian/Bulgarian and the Swedish and the French populations. The similarity between the Armenian and the Bulgarian populations was corroborated by the EAG measurements that showed identical responses from males from the two populations. Among the Hungarian laboratory-reared insects, striking differences in receptor frequencies were observed between antennae from different males. Some of the antennae showed a proportion of Z5-10:OAc sensilla similar to that of the Swedish insects and some showed a proportion similar to the Bulgarian and Armenian insects. These two categories were designated Hungarian type 1 and Hungarian type 2, respectively. A male was assigned to type 1 if it had seven or more Z5-10:OAc sensilla in the sample of 10 and was

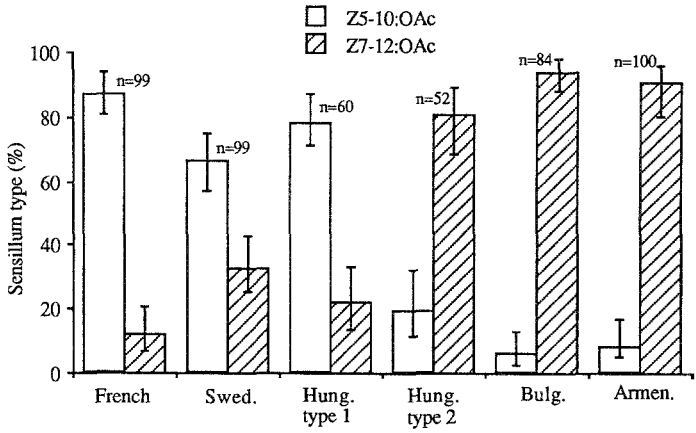

FIG. 1. Percentage of sensilla containing receptors tuned to the female sex pheromone components Z5–10:OAc and Z7–12:OAc in *Agrotis segetum* laboratory cultures of different origin, investigated in this report and in Löfstedt et al. (1986). Vertical bars indicate 95% confidence interval.

assigned to type 2 if it had three or less Z5–10:OAc sensilla in the sample. No insects fell between the groups. The difference between the most similar samples assigned to different groups was statistically significant at the 90% level ($P < 0.1$) according to the Fisher's exact probability test (Siegel, 1956). In all the investigated populations the Z9–14:OAc sensitive sensilla constituted a proportion of about 1% (range 0–2%).

To investigate the Hungarian population of the turnip moth further, males were selected by being caught in traps baited with either the three-component blend, a mixture of Z7–12:OAc and Z9–14:OAc, a mixture of Z5–10:OAc and Z7–12:OAc, pure Z7–12:OAc, or a mixture of Z5–10:OH and Z7–12:OH. The last treatment was included because in a preliminary experiment males caught in traps baited with pure Z7–12:OH, possessed only 19% Z5–10:OAc sensilla (Hungarian type 2). The males caught were typed as Hungarian type 1 or Hungarian type 2.

In the traps baited with the complete three-component mixture, both types of insects were caught, while all insects except three caught on the three different baits lacking Z5–10:OAc in their bait showed a typical type 2 sensillum frequency (Table 2). The field experiments showed clearly that both the Hungarian type 1 and type 2 of *Agrotis segetum* coexist in the field and that the type 1 males require Z5–10:OAc in the blend in order to be attracted. The type 2 males, however, seemed to be attracted to all the baits tested.

TABLE 2. TOTAL NUMBER OF ANIMALS CAUGHT IN TRAPS WITH DIFFERENT BAITS AND TYPE OF 10 RANDOMLY CHOSEN MALES FROM EACH TRAP CATCH (TYPE 1 WITH AT LEAST SEVEN Z5-10:OAc SENSITIVE SENSILLA OR TYPE 2 WITH AT MOST THREE Z5-10:OAc SENSILLA OUT OF 10 INVESTIGATED SENSILLA[a]

Bait (μg)	No. caught	Type 1	Type 2
Z5-10:OAc (20)/Z7-12:OAc (20)/ Z9-12:OAc (20)	143	6	4
Z5-10:OAc (20)/Z7-12:OAc(20)	7	2[b]	2[b]
Z7-12:OAc(20)/Z9-14:OAc (20)	46	2	8
Z7-12:OAc (0.05)	160	1	9
Z5-10:OH (500)/Z7-12:OH (500)	109	0	10

[a]The field trappings were performed May 27–28 and August 1–2, 1989, in Budakeszi, Pest county, Hungary.
[b]Out of 4.

DISCUSSION

The turnip moth is clearly polymorphic with respect to the sex pheromone communication system. The Armenian and Bulgarian insects differ so much from the French ones described earlier that an examination of only the pheromone system would probably have classed the two types as different species. The studies of different populations in this paper and in Löfstedt et al. (1986) imply that the European distribution of *A. segetum* is divided into at least three main pheromone populations: a French population producing more Z5-10:OAc and possessing many Z5-10:OAc receptors, a Swedish population producing less Z5-10:OAc and possessing fewer receptors for this compound, and an Armenian/Bulgarian population producing very little Z5-10:OAc and possessing even fewer receptors for this component. The detailed distribution of the different strains is unknown, but the large areas that the different populations occupy and the coexistence of at least the Swedish and Armenian/Bulgarian populations in Hungary suggest that we are dealing with different, more or less isolated, populations rather than with a cline.

The low pheromone titer and the large variation between batches observed in the GC analysis of the Armenian and Bulgarian gland extracts may be due to the fact that only a few of the extracted females actually contain measurable amounts of pheromone. The quantification of phereomone differences among different populations of *A. segetum* is a delicate task not only because of this but also because of the large number of pheromone component candidates involved. Taking these facts into consideration, the results obtained with dif-

ferent techniques, and by different groups of researchers in different laboratories, are remarkably coherent and consistent.

The dominance of Z7–12 : OAc and Z9–14 : OAc in extracts of Bulgarian *A. segetum* females corresponds to the importance of these two compounds for attraction of Bulgarian males, earlier reported by Subchev et al. (1986). In the case of Z9–14 : OAc we hypothesized that the importance of this compound for the Bulgarian population should be reflected in a larger number of receptors for this compound on the male antenna. This was obviously not true.

Our evidence for the cooccurrence of different *A. segetum* pheromone strains in Hungary could be further corroborated by the analysis of offspring from individual field-collected mated females. The male phenotype of the offspring can be assigned by electrophysiological means and the female phenotype by GC analysis of pheromone gland extracts, preferably from individual insects. Assortive mating under natural circumstances would result in apparent linkage between female pheromone production and male pheromone response. Different pheromone strains of the turnip moth might also coexist in other parts of Europe. Analysis of female moths of Swedish origin demonstrated a high variation in ratios between the homologous acetates among individuals (Löfstedt et al., 1985). As a matter of fact, the samples contained females, which, in hindsight, could have been classified as French type or Armenian/Bulgarian type, whereas other individuals conformed to the average Swedish type (Löfstedt et al., 1982).

The evolution of different pheromone strains, as we see in the turnip moth, requires that the stabilizing selection, which is normally assumed to operate on communication systems, must be overcome. The easiest way to envisage how the variation in a sex pheromone communication system can develop is through geographical isolation of different populations from each other (Mayr, 1963). If populations are separated during an evolutionary time span, the selection pressures acting on them in their respective areas might vary considerably and differences between the populations can build up. The interference from another, or several other species, producing a similar sex pheromone in an isolated part of the species disjunct area, would constitute a strong selective pressure for that part of the population to change its pheromone composition, as individuals would spend a lot of time and energy searching for mates of the wrong species. Another explanation could be that mutations, which influence the sex pheromone production and response, may occur in one area but not in the others and may go to fixation by pure chance (Wright, 1955).

A differentiation of the sex communication system in allopatry would make it plausible that the species populations differentiated enough, both ecologically and with respect to genetic incompatibilities, so that the differences formed in allopatry would persist also when the populations came into sympatry or parapatry. If the differentiation of the populations has proceeded so far that hybrids

produced through interpopulation matings would experience a lower fitness than the parents, the pheromone differences in the sympatric areas could become even more accentuated through reinforcement (Dobzhansky, 1940; Butlin, 1987). This reinforcement could occur even if the differentiation had not reached the level of genetic incompatibility, but remained ecological, based on sex pheromone composition differences. As hybrids in some species have been shown to exhibit an intermediate sex pheromone production and response, the hybrids might suffer a substantial loss in fitness. The only moth species that, so far, has been genetically dissected with respect to sex pheromone production and response, *Ostrinia nubilalis* (Klun and Maini, 1979; Hansson et al., 1987; Roelofs et al., 1987; Löfstedt et al., 1988), has at least three genes involved in the control of differences between strains in the sex pheromone communication. These genes are not linked (Löfstedt et al., 1988), which can make hybrid females produce one ratio and hybrid males respond to a different one. These circumstances could be involved in a reinforcement process based on communication incompatibilities.

Pheromone differences could possibly also develop in a species where the populations never became separate, that is, where they remained in sympatry or parapatry through the whole process of divergence. The populations would then be genetically compatible and continuous, but different selective pressures in different areas would create and maintain the differences between the populations (Littlejohn, 1977). The sympatric/parapatric system relying on pure ecological isolation is, however, very unstable. As soon as the selection pressure was relaxed, and insects of the aberrant populations suffered no loss in fitness by mating with an animal from the original population, the polymorphism would rapidly break down (Littlejohn, 1977). If, however, the selection pressures remained strong long enough for the differentiation of the extremes in the species distribution to proceed far enough, and maybe also become associated with some parallel changes in other ecological factors (for instance, host-plant preference), this system might also lead to a stable polymorphism.

Our evidence that two turnip moth populations coexist in Hungary under similar selection pressures on the sex communication system indicates that the populations today are truly parapatric. The two populations also seem to be fairly well defined reproductively. In traps lacking the Z5–10:OAc, almost only Hungarian type 2 males were caught, while in the traps with the three component mixture both types of males were caught. This raises the possibility that the turnip moth in Europe today is a complex of sibling species with different sex pheromones, as was recently shown in a group of primitive New Zealand tortricids (Foster et al., 1986) and for the dingy cutworm complex, *Feltia jaculifera* (Gn.) (Lepidoptera: Noctuidae) in Canada (Struble et al., 1988). The genetic differences between the populations are still to be investi-

gated to extend the picture of the pheromone system variation in *Agrotis segetum*.

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