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MECHANISMS OF BEHAVIORAL ALTERATIONS OF PARASITOIDS REARED IN ARTIFICIAL SYSTEMS

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Abstract—A high quality of mass reared parasitoids is required for successful biological control of pest insects. Although the phenomenon of behavioral deterioration of parasitoids due to rearing in artificial conditions is well known, its significance is often underestimated, and the underlying mechanisms are poorly investigated. We quantified behavioral alterations of parasitoids reared in an artificial system vs. a natural system and elucidated some of the mechanisms involved. The model systems consisted of apple fruits (natural system) or an artificial diet devoid of apple (artificial system), the herbivore *Cydia pomonella*, and its larval parasitoid *Hyssopus pallidus*, a candidate biological control agent. Two parasitoid strains, one reared for 30 generations in the natural system and one in the artificial system, were compared by using the females' ability to respond to frass from codling moth caterpillars fed on apple fruits (apple-frass). The searching response of parasitoids reared in the artificial system compared to those reared in the natural system was reduced by an average of 53.2%. Gas chromatography–mass spectrometry (GC-MS) analyses of the two types of caterpillars' food and of the two corresponding types of frass showed that 15 compounds were present only in apple fruits and apple-frass, three compounds only in artificial diet and artificial-diet-frass, while four compounds were present in both frass types but not in the food sources. This suggests the presence of a food-derived and a host-derived component in the frass. Results from both bioassays and chemical analyses indicate that the kairomonal activity of the frass is due to both apple fruit and host components. The reduced response of parasitoids reared in artificial conditions might, therefore, be due to a lack of recognition of the apple fruit component. In a further experiment, the two parasitoid strains were reared in the opposite system for one generation. While the response to the host frass was significantly reduced in parasitoids that emerged from the artificial system, it was fully restored in parasitoids that emerged from

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the natural system. This indicates that the behavioral alteration was related to a learning process during ontogenesis rather than to a selection exhibited over generations.

Key Words—*Hyssopus pallidus*, parasitoid, *Cydia pomonella*, apple, frass, mass rearing, diet, artificial system, behavioral alteration, host searching.

INTRODUCTION

The success of inundative biological control with parasitoids depends, among other factors, on an efficient mass rearing of insects, with the purpose of producing large numbers of agents of high quality. Quality in terms of high growth, fecundity, reproduction, and survival has often been studied (Bigler, 1994; Thompson and Hagen, 1999). In contrast, little consideration has been given to quality in terms of behavioral performance, despite its significance for biological control (Noldus, 1989; van Lenteren, 1991). For a good performance after release in the field, parasitoids should maintain natural behavioral traits such as an efficient host searching and parasitization behavior. This implies an appropriate recognition and perception of relevant chemical cues.

It is well known that rearing parasitoids under artificial laboratory conditions might lead to the alteration of several behavioral traits (Bautista and Harris, 1997 and references therein), including the deterioration of host searching behavior (Lewis et al., 1990; Vet et al., 1990; Geden et al., 1992). The dramatic consequences of this quality decline have been described, and some attempts have been undertaken to improve the performance of parasitoids affected by behavioral deterioration with conditioning (Udayagiri, 1996, and references therein; Zaki et al., 1998), with priming to semiochemicals prior to release (Hare and Morgan, 1997), or with field manipulations (Lilley et al., 1994). However, in spite of the recurrence of behavioral alterations (Nordlund, 1998), the mechanisms underlying the phenomenon are still little explored.

The aim of this project was to quantify behavioral changes of insectary-reared parasitoids, and to elucidate the underlying mechanisms. As a study system, we used the parasitic wasp *Hyssopus pallidus* (Askew) (Hymenoptera: Eulophidae), a gregarious larval ectoparasitoid and candidate biological control agent of the codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), a major pest of apple fruits, *Malus domestica* (Borkh.) (Rosaceae). *Hyssopus pallidus* has a high fecundity, a rapid development rate, and a strongly female-biased sex ratio (Zaviezo and Mills, 1999; Tschudi-Rein and Dorn, 2001). This parasitoid species has the special ability to enter infested apples through the calyx or the channel made by the host caterpillar, to irreversibly paralyze and then parasitize the host before it leaves the fruit (Mattiacci et al., 1999). As a consequence, *H. pallidus* has the potential to reduce codling moth population levels in orchards and could be a useful alternative to manual removal of infested fruits (Mattiacci et al., 1999). For successful host

location, adult wasps rely on a strong response to host frass traces on the surface and in the channels of the infested fruit.

In a first experiment, we compared two strains of parasitoids. One was reared on caterpillars fed on apple fruits, i.e., in a "natural system." The other was reared on caterpillars fed on an artificial diet, i.e., in an "artificial system," as a model for a commercial mass rearing. The host searching behavior of the two strains was compared in terms of response of adult females to chemical stimuli from the host frass (host feces and silk). Behavioral bioassays were coupled with the identification of the chemicals in both caterpillars' frass and food sources through solvent extraction and gas chromatography–mass spectrometry.

In a second experiment, we reared the two strains in the opposite system for one generation. The purpose was to study the mechanism of behavioral alteration and its reversibility. Parasitoids from the natural system were maintained during development in the artificial system to test whether behavioral alterations are due to a selection pressure over many generations or whether they occur at an ontogenetical level. *Vice versa*, parasitoids from the artificial system were maintained during development in the natural system to test whether the behavioral deterioration is reversible.

METHODS AND MATERIALS

Insect Rearing. Starting from a single colony of *H. pallidus* (Tschudi-Rein and Dorn, 2001), two strains were obtained by rearing parasitoids under different conditions. An "artificial diet strain" ("AD strain") was obtained in the artificial system and an "apple strain" in the natural system as detailed below. Bioassays were carried out with the 30th–32nd generation after split rearing.

AD strain parasitoids were reared on *C. pomonella* caterpillars fed on a wheat germ-based artificial diet ("AD") devoid of apple fruit material (Huber et al., 1972). Newly hatched first instar caterpillars from a laboratory colony (described by Mattiacci et al., 1999) were placed individually in a plastic box (18 $\times$ 18 $\times$ 10 mm) filled with 3.4 g artificial diet. Boxes were kept at 26 $\pm$ 1°C, 55 $\pm$ 10% RH, and 18:6 LD. After 16–21 days, 5th instars were offered to 4–7 days old mated female parasitoids in glass vials at a ratio of 1 parasitoid/host, with a droplet of honey as food. New adult parasitoids emerged 14–17 days later. Upon emergence, they were removed from the vials containing the dead host caterpillar, transferred into Plexiglas cages (25 $\times$ 25 $\times$ 25 cm), and maintained with water and undiluted honey. During parasitization and development, parasitoids were not exposed to plant cues.

Apple strain parasitoids were reared on *C. pomonella* larvae fed on organically grown apple fruits (cv "Bohnäpfel"). For infestation, first instars were placed on the surface of apples. Infested fruits were kept under the same conditions as infested

AD-boxes. After 16–21 days, apples were carefully dissected. Fifth instars were offered to the parasitoids as described for the AD strain. To provide parasitoids with chemical cues of the natural host habitat, a slice of apple fruit (approx. 30 mg) was added to the vials.

Bioassay. Bioassays were performed with 4–7 day old mated parasitoid females. Immediately prior to the bioassay, parasitoids were transferred individually from the emergence cages into glass vials (2 cm diam, 0.8 cm height). One half of a circular filter paper (Whatman, 1.3 cm, grade 1, CAT No. 1001013; Merck, Dietikon, Switzerland) was treated with 20 μl of solvent extracts, and left uncovered for 3 min to allow complete evaporation of the solvent. Subsequently, it was transferred onto a glass petri dish (5 cm diam). The open end of the glass vial with the wasp was positioned over the cue source. The observation started when the parasitoid began to move in the arena and ended 10 min later. The behavioral parameter *searching*, defined as intensive antennal examination on the filter paper, was recorded. The total searching time was measured with a stopwatch by accumulating periods of behavioral activity. Bioassays were conducted from 11:00 a.m. to 17:00 p.m. under natural daylight conditions (900–1800 lux), at 21 $\pm$ 1°C and 50 $\pm$ 5% RH.

Chemical Analyses. Chemical analyses were performed with a gas chromatograph (Hewlett-Packard 6890, Atlanta, USA), equipped with an HP-1 cross-link methyl silicone capillary column (30 m, 0.25 mm internal diam, 0.25 μm film thickness). This was coupled to a mass-spectrometer (Hewlett-Packard 5973, Atlanta, USA) with a mass selective FID-detector. Helium (purity $\geq$ 99.99%) at a constant pressure of 90 kPa was used as a carrier gas. Samples of 1 μl were injected manually in a splitless mode at an initial inlet temperature of 250°C. The oven temperature was held at 50°C for 2 min, then increased to 300°C at a rate of 8°C/min, and then held at 300°C for 15 min. A solvent delay of 4.5 min was programmed, and a purge flow of 50 ml/min was started after 2 min. Compounds were identified by comparison of the mass spectra with those in the mass spectra database NIST98 and in our own library. The identity of compounds was confirmed by coinjection of authentic samples. Synthetic compounds were obtained from Fluka Chemie AG (Buchs, Switzerland) and were $\geq$99.5–99.8% pure. For quantification, the internal standards *trans*-4-decen-1-al (IS1) and *cis*-11-hexadecen-1-yl acetate (IS2) were added to the sample at a ratio of 20 ng/μl. To improve detectability of compounds present in very small amounts, samples were concentrated tenfold and reinjected.

Frass Extracts. Preliminary experiments were conducted to obtain a bioactive frass extract. Frass material expelled from infested apples was air dried at 55 $\pm$ 10% RH and at room temperature for 5 days. Frass was subsequently extracted for 18 hr with solvents of increasing polarity: *n*-hexane, diethyl ether, acetone, and methanol (purity $\geq$99.5–99.8%). The four extracts were used for the bioassay described above to monitor the bioactivity. Additionally, dose-response tests were carried out for each extract. As a control, the bioactivity of fresh host frass expelled

by fifth instars feeding on apple fruits was tested. The most active extract was the one obtained with diethyl ether, followed by hexane.

Attempts to isolate the active component of the frass failed, as fractions made with mixtures of diethyl ether and hexane with increasing proportions of diethyl ether were all bioactive, though their chemical composition was different (data not shown). For all subsequent experiments the diethyl ether extract was used. The chemical composition of this extract was characterized by GC-MS as described above.

Comparison of the Two Parasitoid Strains. Cue sources. Parasitoids of both strains were offered diethyl ether extracts from "apple-frass" produced by caterpillars feeding on apple fruits, "AD-frass" produced by caterpillars feeding on artificial diet, and diethyl ether (purity $\geq$99.8%) as a solvent control. As differences were found between the response of the two strains to these cue sources, parasitoids were subsequently tested with diethyl ether extracts from apple fruit and artificial diet, and diethyl ether as solvent control. For both experimental sets, 20 female wasps were tested for each treatment and strain. Differences between strains and treatments were tested with two-way analysis of variance, followed by Student–Newman–Keuls pairwise multiple comparison (Zar, 1999).

Extractions. Both frass types were air dried for 2 days and extracted in diethyl ether (purity $\geq$99.8%) at a ratio of 0.13 g/ml solvent. Freshly prepared artificial diet was extracted at a ratio of 0.2 g/ml solvent, considering the fresh/dry ratio of 1/0.65 (w/w). For the preparation of the apple fruit extract, skin, pulp, and seeds were first extracted separately, as these organs are likely to have a different chemical composition (Guadagni et al., 1971). For the extracts, 0.2 g/ml of fresh skin and fresh pulp were grated directly in to diethyl ether, while seeds were cut in thin slices of 0.2–0.3 mm. The three apple organs were recombined to obtain an extraction with similar chemical composition to that of the apple component of the apple frass. The final extract was made from a mixture of 8.75% skin, 85% pulp, and 6.25% seeds at a ratio of 0.2 g/ml. On the basis of preliminary dose-response experiments, extracts from both food sources were concentrated tenfold in order to elicit a high response.

Mechanisms of Behavioral Alterations. Female parasitoids from the apple strain were transferred into the artificial system for parasitization, i.e., they were allowed to parasitize a caterpillar that had been feeding on artificial diet. The offspring was maintained in the artificial system throughout development. *Vice versa*, female parasitoids from the AD strain were transferred into the natural system, and their offspring was maintained in this system during development. The behavior of emerging adults was assessed with the bioassay described above, by testing the response of females to an extract of apple-frass. Both parasitoids from the apple strain and from the AD strain reared in their original system were used as control. For each treatment, 20 wasps were tested. Data were analyzed by two-way ANOVA followed by Bonferroni method for pairwise comparison (Zar, 1999).

RESULTS

The average amount of frass produced by one fifth-instar *C. pomonella* caterpillar during 24 hr was 60.5 ± 4.1 mg (mean ± SE, $N = 10$). Fresh frass and diethyl ether extracts of frass from caterpillars feeding on apple fruits were both attractive for *H. pallidus*. The highest response to frass extract was obtained with 20 μl of extract containing an amount of frass equivalent to that produced by a caterpillar in approx. 2 hr. Chemical analyses of the frass extract revealed 36 major compounds including fatty acids, *n*-alkanes, terpenes, sterols, aldehydes, fatty esters, and tocopherols (Table 1).

Comparison of the Two Parasitoid Strains. Extracts from the three apple fruit organs differed in their chemical composition (Table 2). The skin contained the majority of compounds, and in the highest quantity. The pulp contained only few compounds in relatively low quantities. Alpha-farnesene and esters were found only in the skin. Fatty acids and *n*-alkanes were detected mainly in the skin, only traces in other fruit parts. Sterols and tocopherols, on the contrary, were typical of seeds, except for β-sitosterol, which was mainly found in the skin. Squalene was found in all three apple fruit organs.

The apple-frass extract and the AD-frass extract differed in their chemical composition (Figure 1). Several compounds from apple-frass were not detected in AD-frass and *vice versa*. The terpene α-farnesene, the four aldehydes, 2-heptenal, nonanal, decanal, and 2,4-decadienal, and the five *n*-alkanes, *n*-heneicosane, *n*-tricosane, *n*-pentacosane, *n*-octacosane, and *n*-hentriacontane were found exclusively in apple-frass and apple fruits. Sorbic acid, methylparaben, and cholesterin were found only in AD-frass and artificial diet. Gamma-sitosterol, stigmastenone, and two unknown compounds were found in both frass types and not in the caterpillars' food sources. Both unknown compounds were also detected in diethyl ether extracts from silk of *C. pomonella* (data not shown).

Both types of caterpillar frass and both types of caterpillar food source elicited significant searching activity of parasitoids. However, the response of the two parasitoid strains differed depending on the frass type or food type offered as chemical stimuli (Figure 2). Apple strain parasitoids searched nearly twice as long (188%) on apple-frass as the AD strain (Mann–Whitney $t = 156.5$, $P < 0.01$, $N = 20$), while AD strain parasitoids searched as long on apple-frass as on AD-frass ($t = -0.241$, $P = 0.811$, $N = 20$) (Figure 2.1). Apple strain parasitoids showed a much stronger response to apple fruit cues than AD strain parasitoids (Kruskal–Wallis ANOVA $p = 3$, $q = 8.935$, $P < 0.05$) (Figure 2.2). *Vice versa*, AD strain parasitoids had a significantly higher response to artificial diet cues than to apple fruit cues (Kruskal–Wallis ANOVA $p = 2$, $q = 3.564$, $P < 0.05$).

Mechanisms of Behavioral Alterations. Parasitoids from the apple strain were reared for one generation in the artificial system. The response of emerged females to apple-frass was reduced on average by 49.2% (two-way ANOVA, Bonferroni,

TABLE 1. MAJOR COMPOUNDS (IN ng [a]) FOUND IN 1 μl DIETHYL ETHER APPLE-FRASS EXTRACT

Chemical class	Peak[b]	Compound	Apple-frass extract
Aldehydes	1	2-Heptenal	2.202 ± 1.123
	3	Nonanal	0.879 ± 0.511
	5	Decanal	0.303 ± 0.163
	6	2-Decenal	2.055 ± 1.142
	7	2.4-Decadienal	2.564 ± 1.702
Terpenes	10	α-Farnesene	48.801 ± 22.34
	32	Squalene	9.903 ± 6.335
n-alkanes	18	*n*-Heneicosane (*n*-C21)	0.558 ± 0.299
	22	*n*-Docosane (*n*-C22)	3.667 ± 1.997
	24	*n*-Tricosane (*n*-C23)	2.571 ± 1.327
	27	*n*-Tetracosane (*n*-C24)	3.885 ± 1.963
	28	*n*-Pentacosane (*n*-C25)	4.012 ± 2.331
	29	*n*-Hexacosane (*n*-C26)	7.115 ± 4.015
	30	*n*-Heptacosane (*n*-C27)	9.903 ± 4.982
	31	*n*-Octacosane (*n*-C28)	5.794 ± 3.258
	33	*n*-Nonacosane (*n*-C29)	50.036 ± 27.77
	34	*n*-Triacontane (*n*-C30)	4.030 ± 2.236
	36	*n*-Hentriacontane (*n*-C31)	4.709 ± 2.558
	39	*n*-Dotriacontane (*n*-C32)	1.952 ± 0.885
Fatty acids	2	Hexanoic acid	0.303 ± 0.201
	15	Palmitic acid	12.279 ± 7.159
	19	Linoleic acid	113.545 ± 61.52
	20	Elaidic acid	70.412 ± 38.26
	21	Stearic acid	22.861 ± 16.55
Fatty esters	8	Hexanoic acid hexyl ester	0.758 ± 0.410
	11	Fatty acid ester I	0.133 ± 0.088
	14	Palmitic acid methyl ester	0.030 ± 0.024
	17	Linoleic acid methyl ester	0.576 ± 0.287
	23	Fatty acid ester II	0.485 ± 0.256
	26	Fatty acid ester III	4.042 ± 2.203
Tocopherols	35	γ-Tocopherol	0.418 ± 0.258
	38	α-Tocopherol	1.036 ± 0.866
Unknown	40	Unknown I	53.515 ± 22.26
	41	Unknown II	89.103 ± 40.25
Sterols	42	γ-Sitosterol	15.448 ± 9.933
	45	Stigmastenone	0.303 ± 0.185

[a] All samples were analyzed in triplicate and are expressed as mean values (± SE).
[b] Peak number corresponds to that of Figure 1.

$t = 6.296$, $P < 0.05$, $N = 20$) (Figure 3). The response was as low as that of AD strain parasitoids reared for 32 generations in the artificial system (two-way ANOVA, Bonferroni, $t = -0.091$, ns, $N = 20$). *Vice versa*, parasitoids from the AD strain were reared for one generation in the natural system. The response of

TABLE 2. MAJOR COMPOUNDS (IN ng [a]) FOUND IN 1 μl DIETHYL ETHER EXTRACT OF APPLE FRUIT PREPARED FROM PULP, SEEDS, AND SKIN IN THE RATIO 0.85:0.0625:0.0875, AND CORRESPONDING VALUES FOR EACH OF THE THREE APPLE ORGANS

Chemical class	Peak[b]	Compound	Recomposed apple fruit extract	Apple organs		
				Pulp	Seeds	Skin
Aldehydes	1	2-Heptenal	0.098 ± 0.043	—	0.098 ± 0.043	—
	3	Nonanal	0.018 ± 0.005	—	—	0.018 ± 0.005
	5	Decanal	0.071 ± 0.026	—	—	0.071 ± 0.026
	7	2,4-Decadienal	0.034 ± 0.021	—	—	0.034 ± 0.021
Terpenes	10	α-Farnesene	1.999 ± 0.722	—	—	1.999 ± 0.722
	32	Squalene	3.875 ± 1.875	0.591 ± 0.302	0.819 ± 0.424	2.465 ± 1.246
n-alkanes	13	*n*-Nonadecane (*n*-C19)	0.029 ± 0.014	—	—	0.029 ± 0.014
	16	*n*-Eicosane (*n*-C20)	0.044 ± 0.019	—	—	0.044 ± 0.019
	18	*n*-Heneicosane (*n*-C21)	0.098 ± 0.058	—	—	0.098 ± 0.058
	24	*n*-Tricosane (*n*-C23)	0.216 ± 0.153	—	—	0.216 ± 0.153
	28	*n*-Pentacosane (*n*-C25)	0.461 ± 0.197	—	—	0.461 ± 0.197
	30	*n*-Heptacosane (*n*-C27)	9.056 ± 5.117	—	—	9.056 ± 5.117
	31	*n*-Octacosane (*n*-C28)	1.866 ± 0.887	—	—	1.866 ± 0.887
	33	*n*-Nonacosane (*n*-C29)	52.238 ± 31.41	0.178 ± 0.088	0.061 ± 0.032	51.999 ± 29.59
	36	*n*-Hentriacontane (*n*-C31)	0.551 ± 0.289	—	—	0.551 ± 0.289
Fatty acids	2	Hexanoic acid	0.004 ± 0.003	—	0.004 ± 0.003	—
	12	Myristic acid	0.074 ± 0.045	—	—	0.074 ± 0.045
	15	Palmitic acid	1.116 ± 0.785	—	0.018 ± 0.011	1.098 ± 0.569
	19	Linoleic acid	5.043 ± 3.214	—	—	5.043 ± 3.214
	20	Elaidic acid	1.817 ± 1.106	—	—	1.817 ± 1.106
	21	Stearic acid	1.751 ± 0.697	—	—	1.751 ± 0.697
	25	Eicosanoic acid	0.724 ± 0.385	—	—	0.724 ± 0.385
Fatty esters	8	Hexanoic acid hexyl ester	0.129 ± 0.078	—	—	0.129 ± 0.078
	14	Palmitic acid methyl ester	0.142 ± 0.098			0.142 ± 0.098
	17	Linoleic acid methyl ester	0.151 ± 0.076	—	—	0.151 ± 0.076

Tocopherols	35	γ-Tocopherol	0.134 ± 0.094	—	0.134 ± 0.094	—
	38	α-Tocopherol	0.148 ± 0.101	—	0.148 ± 0.101	—
Sterols	43	Stigmasterol	0.036 ± 0.021	—	0.036 ± 0.021	—
	44	β-Sitosterol	6.291 ± 2.789	—	0.696 ± 0.315	5.594 ± 2.222

[a] All samples were analyzed in triplicate and are expressed as mean values (± SE).
[b] Peak number corresponds to that of Figure 2.

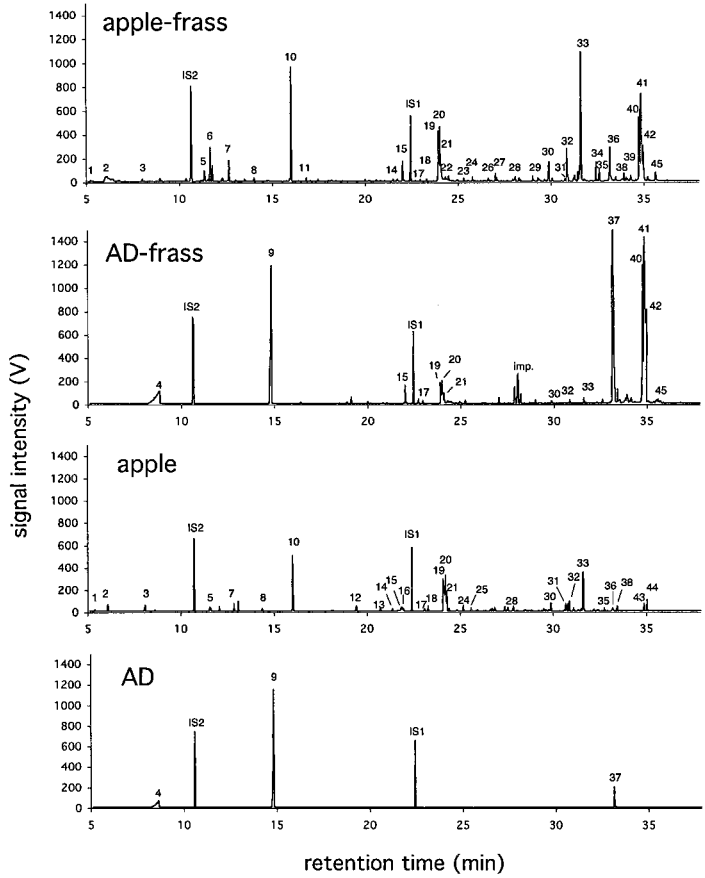

FIG. 1. Total ion chromatograms of diethyl ether extracts from apple-frass, artificial-diet-frass (AD-frass), apple fruit, and artificial diet (AD). Numbered peaks correspond to the following compounds: (1) 2-heptenal[a], (2) hexanoic acid[a], (3) nonanal[a], (4) sorbic acid[m], (5) decanal[a], (6) 2-decenal, (7) 2,4-decadienal[a], (8) hexanoic acid hexyl ester[a], (9) methylparaben[m], (10) α-farnesene[a], (11) unidentified fatty acid ester I, (12) myristic acid, (13) *n*-nonadecane (*n*-C19), (14) palmitic acid methyl ester[a], (15) palmitic acid, (16) *n*-eicosane (*n*-C20), (17) linoleic acid methyl ester, (18) *n*-heneicosane (*n*-C21)[a], (19) linoleic acid, (20) elaidic acid, (21) stearic acid, (22) *n*-docosane (*n*-C22), (23) unidentified fatty acid ester II, (24) *n*-tricosane (*n*-C23)[a], (25) eicosanoic acid, (26) unidentified fatty acid ester III, (27) *n*-tetracosane (*n*-C24), (28) *n*-pentacosane (*n*-C25)[a], (29) *n*-hexacosane (*n*-C26), (30) *n*-heptacosane (*n*-C27), (31) *n*-octacosane (*n*-C28)[a], (32) squalene, (33) *n*-nonacosane (*n*-C29), (34) *n*-triacontane (*n*-C30), (35) γ-tocopherol[a] (36) *n*-hentriacontane (*n*-C31)[a], (37) cholesterol[m], (38) α-tocopherol[a], (39) *n*-dotriacontane (*n*-C32), (40) unknown I[f], (41) unknown II[f], (42) γ-sitosterol[f], (43) stigmasterol, (44) β-sitosterol, (45) stigmastenone[f]. Compounds detected exclusively in apple fruit and apple-frass[a]; compounds detected exclusively in AD and AD-frass[m]; compounds detected exclusively in apple-frass and AD-frass[f]. IS1: *trans*-4-decen-1-al, IS2: *cis*-11-hexadecen-1-yl acetate.

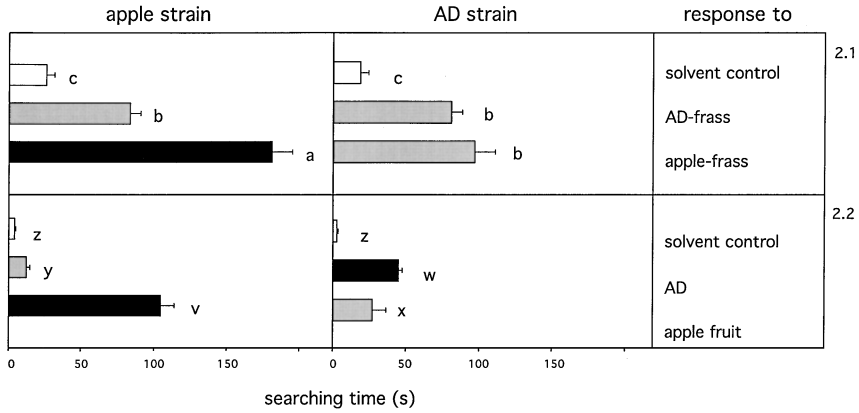

FIG. 2. Response of parasitoids from the apple strain and artificial diet (AD) strain to (2.1) diethyl ether extracts of both frass types, apple-frass and AD-frass, and (2.2) to diethyl ether extracts of both hosts' food types, apple fruit and AD. Bars indicate the average time (±SE) spent searching on each treatment in single-choice bioassays. Data from frass extracts and from food extracts were analyzed separately. Letters indicate significant differences among strains and treatments ($N = 20$).

emerged females to apple frass was increased on average by 235.3% (two-way ANOVA, Bonferroni, $t = 8.376$, $P < 0.05$, $N = 20$). This response reached the original level obtained with apple strain parasitoids reared for 32 generations in the natural system (two-way ANOVA, Bonferroni, $t = -2.170$, ns, $N = 20$). The response of parasitoids was affected only by the system in which they developed, and not by the strain of their mother (two-way ANOVA strain: $F = 2.56$, $P = 0.114$; treatment: $F = 107.63$, $P < 0.001$).

DISCUSSION

The ability of the parasitoid *H. pallidus* to find its host *C. pomonella* upon release in the field depends, among other factors, on an optimal response to host frass. In fact, females find caterpillars concealed in the fruit mainly by detecting frass on the surface of the fruit and by following frass traces inside the fruit (Mattiacci et al., 1999). The present study shows a strong response of *H. pallidus* to both fresh apple-frass and to frass extracts prepared with diethyl ether. This underlines the fundamental role of chemical stimuli in the frass for triggering a response of parasitoids. Unlike in other systems, where active mixtures elicited lower responses of parasitoids compared to the original cue sources (Turlings et al., 1990; Dutton et al., 2000), here, frass extracts maintained the bioactivity of fresh frass material.

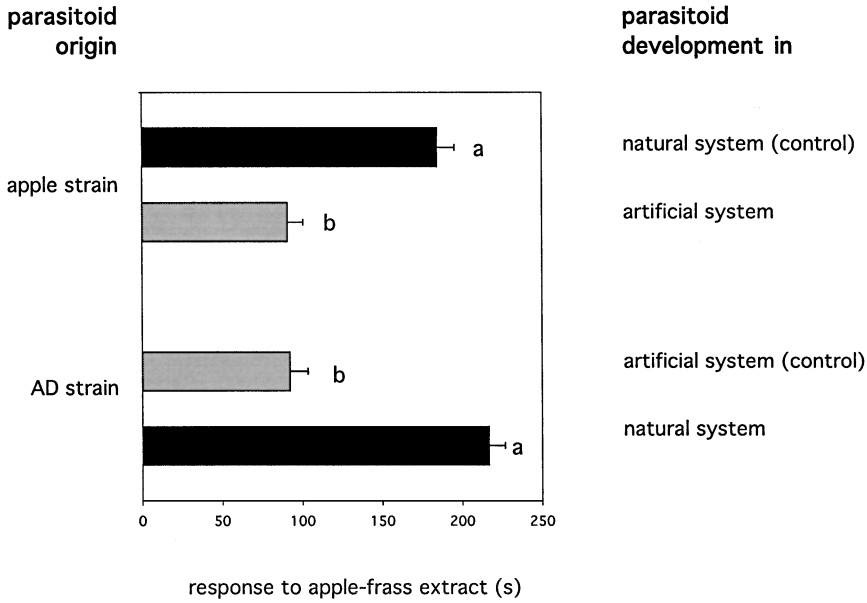

FIG. 3. Deterioration of the behavioral response of *H. pallidus* females to host frass cues during ontogenesis: response of parasitoids from the apple strain after developing in the artificial vs. the natural system. Reversibility of the behavioral deterioration during ontogenesis: response of parasitoids from the artificial diet strain (AD strain) after developing in the natural vs. the artificial system. In the natural system, apple fruits were used for rearing the host *C. pomonella*; in the artificial system, apple fruits were replaced by an artificial diet devoid of apple cues. Bars indicate the average time (±SE) spent searching on a diethyl ether apple-frass extract. Letters indicate significant differences among parasitoid groups ($N = 20$).

The bioactivity of frass for *H. pallidus* was affected by the caterpillar's food. Apple strain parasitoids preferred frass from apple-feeding hosts to frass from AD-feeding hosts. This indicates that apple-related compounds in the frass play an important role in eliciting a response of parasitoids. The significance of the host plant for the kairomonal activity of the herbivore's frass has been shown in different systems (Ramachandran et al., 1991; Eller et al., 1992; Thibout et al., 1995).

The present study demonstrates that the conditions in which parasitoids are reared are decisive for the behavioral performance of emerging adults. The comparison of the two parasitoid strains reveals a marked difference between the natural and the artificial system. Parasitoids reared in the natural system showed a high response to both apple and apple-frass cues. This is consistent with the results of many studies, providing ample evidence of the high innate response of parasitoids

to both the food plants of their hosts (Udayagiri and Jones, 1992; Cortesero et al., 1993; Dutton et al., 2002) and the plant-host complex (Vet and Dicke, 1992). Surprisingly, parasitoids reared in the artificial system without apple showed a dramatically reduced response to both apple and apple-frass cues. This may negatively affect their performance in biological control programs, in terms of habitat location and host location, respectively. The present investigation suggests that the response of this parasitoid species towards the plant–host complex is not entirely innate, but there is a phenotypic plasticity towards chemicals in the rearing environment. The behavioral response of the adult wasps becomes, therefore, an important parameter for assessing the quality of parasitoids, and a valuable tool for controlling the suitability of the rearing procedure. Chemical analyses of caterpillar frass and food sources revealed that many compounds in the apple-frass were present only in apple fruits, suggesting their plant origin. Similarly, some compounds in the AD-frass were present only in AD, suggesting their origin from the artificial diet. Other compounds were found in both types of frass, but not in the caterpillar food sources. These compounds are likely to be related to the host caterpillar. Most compounds identified in the apple-frass, such as terpenes and the *n*-alkanes, were also identified in apple skin. However, tocopherols and one sterol were only detected in apple seeds. The caterpillar feeds on the apple skin, then penetrates into the fruit, proceeds to its center, and eats the seeds. Our data indicate that initial and late feeding stages are of particular significance for the chemical composition of the caterpillar frass. The two terpenes and the three dominant *n*-alkanes that we identified in both apple fruit and apple-frass have previously been reported from apple plants and fruits (Dutton et al., 2000, 2002; Hern and Dorn, 2001). The two tocopherols that we identified in apple fruit and apple-frass have been detected recently in apple fruits (Schmitz and Noga, 2001). Unlike the two phytosterols β-sitosterol and stigmasterol, which we found only in the apple fruits, and likely to be metabolized and transformed by caterpillars (Svoboda and Lusby, 1994), γ-tocopherol and α-tocopherol may have been excreted by *C. pomonella* larvae directly. Further, the two unknown compounds that we identified exclusively in the two frass types were found to be dominant compounds of the silk of *C. pomonella* larvae (data not shown). These results indicate two categories of compounds in the frass: compounds derived from the caterpillar food (food components), ingested and released in the frass, and compounds derived from the caterpillar itself (host components). Behavioral experiments provide evidence that the kairomonal activity of the frass is due to the activity of both components. The low response of AD strain parasitoids to apple-frass is probably due to a recognition of host components only, but not of apple components. The high response of apple strain parasitoids is probably the result of a recognition of both apple and host components. Fruit cues are, therefore, not only important for host handling and oviposition behavior (Mattiacci et al., 2000), but also for promoting host finding in this species.

Further experiments were carried out to analyze whether the observed behavioral alterations are a direct consequence of the chemical composition of the rearing environment. In particular, we investigated whether the individual experience of parasitoids with fruit cues during development may be solely responsible for their response to host frass cues as adults. When parasitoids from the natural system were exposed to the artificial system during development, emerged adults showed a dramatic reduction in response. This indicates that behavioral deterioration was due to a lack of individual experience with apple cues during ontogenesis and not to a selection pressure. *Vice versa*, when parasitoids from the artificial system were exposed to the natural system during development, adult parasitoids showed the original high level of response. An experience with apple cues during ontogenesis was sufficient to revert completely the previous behavioral deterioration. The option to restore host searching ability within the developmental time of a single generation opens interesting perspectives for the quality management of *H. pallidus*.

In many parasitoid species, the response to host-related cues is considered to be genetically fixed. In contrast, the response to plant-related cues is considered flexible and subject to learning (Lewis and Tumlinson, 1988; Vet and Dicke, 1992). A remarkably high genetic component has been found for the response of the parasitoid *Cotesia glomerata* (L.) (Hymenoptera: Braconidae) to *Pieris brassicae* (L.) (Lepidoptera: Pieridae) infested cabbage plants, i.e., to an unseparated host–plant complex (Gu and Dorn, 2000). The present study indicates that the response of *H. pallidus* to the host components of frass might be genetically fixed. The response to the apple components of frass, however, seems to depend on the mode of parasitoid rearing, and suggests a potential impact of learning. Parasitoids may have learned the apple components of the frass through association of apple cues with the presence of the host, a process that is expected to occur during emergence or in the early adult stage (Kester and Barbosa, 1991; Turlings et al., 1993; Storeck et al., 2000). However, unlike in many cases of classical conditioning (Kerguelen and Cardé, 1996), the association did not require a reward in terms of oviposition. Further, experimental evidence suggests a potential influence of chemical stimuli experienced in rearing on the degree of adult response to the same stimuli. Coincidently, parasitoids exposed to apple cues during rearing responded strongly to apple cues as adults, and parasitoids exposed to artificial diet cues responded strongly to artificial diet cues. This is reminiscent of a process of sensitization, in which continuous exposure to chemical cues may have enhanced and reinforced the adult response to these cues. However, sensitization *sensu* Papaj and Prokopy (1989) presumes an innate response to the stimulus that is not expected for artificial diet. Further work is in progress to define the learning mechanisms involved in detail and particularly to elucidate when the parasitoids learn the chemical stimuli that influence their behavior as adults.

In conclusion, the present study highlights the significance of plant cues in the rearing system for the behavioral quality of the emerging parasitoids. It further shows that, even after more than 30 generations of rearing under artificial conditions, the required response of parasitoids to important chemical cues from their hosts' habitat can be restored within a single generation, indicating that learning of plant-derived stimuli takes place during the parasitoid ontogenesis.

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