1.5 ml microcentrifuge tube, containing 0.5 ml of ultrapure water (Merck, Darmstadt, Germany). The females sank to the bottom of the tube. They were then incubated at 4-6 °C and the supernatant was used 2-18 days later.

Water extracts from gelatinous matrices, removed from white, living females with their hind ends above the agar surface, were obtained by incubating 12 matrices for 2 days at 4-6 °C in 200 µl ultrapure water. Water extracts from females from which the gelatinous matrices had been removed were compared with the extracts from gelatinous matrices.

Solvent extracts from females were obtained by incubating twelve white, intact females for 22 h in 200 µl n-hexane, ethyl acetate, methylene chloride, methanol, or ultrapure water (all from Merck; the polarity increases from n-hexane to water) at 4-6 °C. Solvents without females served as controls.

Enzyme treatments of female extracts

The supernatants of aqueous extracts from females were incubated for 20.5 h at 37 °C with 10 mg/ml pronase or with 125 U/ml cytosolic leucine aminopeptidase (both from Sigma). Supernatants of aqueous extracts from females and enzyme solutions alone served as controls.

Vanillic acid treatments

Vanillic acid (Sigma) was solubilized in a concentration of 1 mmol/l in acetone (Merck). A 1 %, 0.1 %, or 0.01 % solution of this stock solution in distilled water (final vanillic acid concentration 10 µmol/l, 1 µmol/l, or 100 nmol/l, respectively) was used in the bioassay. Water extracts from females were used as controls.

Bioassay system

The attractiveness for *H. schachtii* males of the solutions obtained as described above was tested in a bioassay system. The bioassay was performed in sterile 6 cm diameter plastic Petri dishes, coated with 1.5 ml of 1.5 % (w/v) autoclaved agarose (Type I, Sigma) in glass-distilled water. Eight µl of the aqueous test solutions were pipetted onto a 5 mm diameter filter-paper disk (cut from paper no. 595, Schleicher & Schuell, Dassel, Germany) placed on the agarose surface in the centre of the Petri dish. Eight µl of the organic solvent extracts and solvent controls were pipetted also onto 5 mm diameter filter-paper disks. After solvent evaporation, the filter-paper disks were transferred onto the agarose surface, and 8 µl of ultrapure water were added. All test solutions were allowed to diffuse into the agarose for 3 h before the males were added to the plates. A 7.5 mm radius circle with its centre in the centre of the filter-paper disk was marked on the bottom of the Petri dish; the distance between the circle and the paper margin was thus 5 mm.

Three males were transferred per Petri dish with a fine needle equidistantly onto the agarose above the circle.

Table 1. Effects of different solvent extracts and enzymatically treated water extracts from *Heterodera schachtii* females on males.

Treatment	Males tested	Mobile males	Males with filter-paper contact, time (min)				
			10	30	60	90	120
n-Hexane extract	54	54	14 (25.9 %) **	22 (40.7 %) ***	24 (44.4 %) ***	25 (46.3 %) ***	23 (42.6 %) ***
n-Hexane	54	54	2 (3.7 %) **	1 (1.9 %) ***	2 (3.7 %) ***	3 (5.6 %) ***	2 (3.7 %) ***
Ethyl acetate extract	54	54	12 (22.2 %) **	22 (40.7 %) ***	31 (57.4 %) ***	31 (57.4 %) ***	34 (63 %) ***
Ethyl acetate	54	54	2 (3.7 %) **	2 (3.7 %) ***	1 (1.9 %) ***	3 (5.6 %) ***	2 (3.7 %) ***
Methylene chloride extract	54	54	22 (40.7 %) ***	33 (61.1 %) ***	37 (68.5 %) ***	38 (70.4 %) ***	34 (63 %) ***
Methylene chloride	54	54	0 ***	1 (1.9 %) ***	2 (3.7 %) ***	2 (3.7 %) ***	2 (3.7 %) ***
Methanol extract	54	54	11 (20.4 %) **	19 (35.2 %) ***	23 (42.6 %) ***	27 (50 %) ***	26 (48.1 %) ***
Methanol	54	54	1 (1.9 %) **	1 (1.9 %) ***	0 ***	0 ***	2 (3.7 %) ***
Water extract	54	53	11 (20.8 %) **	19 (35.8 %) ***	22 (41.5 %) ***	19 (35.8 %) ***	20 (37.7 %) ***
Water	54	53	1 (1.9 %) **	3 (5.7 %) ***	2 (3.8 %) ***	3 (5.7 %) ***	2 (3.8 %) ***
Water extract plus pronase	84	84	22 (26.2 %) *	53 (63.1 %) ***	50 (59.5 %) ***	60 (71.4 %) ***	59 (70.2 %) ***
Pronase	84	84	9 (10.7 %) *	3 (3.6 %) ***	5 (6 %) ***	1 (1.2 %) ***	1 (1.2 %) ***
Water extract	84	84	20 (23.8 %)	35 (41.7 %)	39 (46.4 %)	42 (50 %)	44 (52.4 %)
Water extract plus leucine aminopeptidase	84	84	36 (42.9 %) ***	68 (81 %) ***	70 (83.3 %) ***	70 (83.3 %) ***	68 (81 %) ***
Leucine aminopeptidase	84	84	6 (7.1 %) ***	9 (10.7 %) ***	8 (9.5 %) ***	6 (7.1 %) ***	6 (7.1 %) ***
Water extract	84	84	28 (33.3 %)	55 (65.5 %)	59 (70.2 %)	62 (73.8 %)	58 (69 %)

* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; according to the Chi-square test with Yates correction.

Their behaviour towards the test solutions was observed after 10, 30, 60, 90, and 120 min with the aid of a stereo-microscope. The number of males that had contacted the filter paper disk at each given time was recorded. The bioassays were performed four to five times with a total of 60 (water extracts from females and their gelatinous matrices), 18 (extracts from females with different solvents), 28 (enzymatically treated extracts from females), and 18-20 (vanillic acid experiments) Petri dishes per treatment. The results were statistically analyzed with the Chi-square test with Yates correction.

Results

Table 1 shows that the extracts from *H. schachtii* females with n-hexane, ethyl acetate, methylene chloride, methanol, and water attracted significantly more males than the corresponding solvent controls from 10 to 120 min after beginning of the test. The highest male attraction rates of the solvent controls were found after 90 min with water (5.7 %), n-hexane (5.6 %) and ethyl acetate (5.6 %). The methylene chloride extracts showed the highest attraction rates of the solvents tested (70.4 % after 90 min).

Water extracts from females incubated with pronase and leucine aminopeptidase attracted significantly more males than the enzyme controls at every observation time (Table 1). In tests with pronase the highest attraction rates were 71.4 % (90 min after beginning of the test) and 10.7 % for the corresponding enzyme controls (10 min). In tests with leucine aminopeptidase attraction rates reached 83.3 % (60 and 90 min) and 10.7 % for the enzyme controls (30 min).

When compared with the water extracts, vanillic acid had no attraction effects (Table 2). From 30 to 120 min after beginning of the test, the lack of attraction to vanillic acid treatments compared with the attraction to extracts from females were highly significant. With the exception of 1 µmol/l vanillic acid, no significant differences were noted at the first observation time.

Table 2 further shows that the percentage of males attracted by gelatinous matrices and extracts from females from which the matrices had been removed did not differ. The attraction percentages for extracts from females ranged from 10.6 % to 43.3 % with a maximum 90 min after the beginning of the test. The corresponding percentages for the extracts from gelatinous matrices ranged from 13.3 % to 42.2 % with a maximum at the end of the test.

Discussion

This study shows that the substances with sex pheromone activity produced by *H. schachtii* females are pre-

Table 2. Comparison of the effects of vanillic acid and water extracts from *Heterodera schachtii* females and of water extracts from females and their gelatinous matrices on males.

Treatment	Males tested	Mobile males	Males with filter-paper contact, time (min)				
			10	30	60	90	120
Vanillic acid, 100 nmol/l	60	60	2 (3.3 %) ns	1 (1.7 %) ***	1 (1.7 %) ***	1 (1.7 %) ***	3 (5 %) ***
Water extract from females	60	60	9 (15 %) ns	18 (30 %) ***	29 (48.3 %) ***	40 (66.7 %) ***	36 (60 %) ***
Vanillic acid, 1 µmol/l	60	60	5 (8.3 %) ***	4 (6.7 %) ***	1 (1.7 %) ***	2 (3.3 %) ***	4 (6.7 %) ***
Water extract from females	60	60	29 (48.3 %) ***	38 (63.3 %) ***	44 (73.3 %) ***	48 (80 %) ***	40 (66.7 %) ***
Vanillic acid, 10 µmol/l	54	54	2 (3.7 %) ns	4 (7.4 %) ***	3 (5.6 %) ***	5 (9.3 %) ***	5 (9.3 %) ***
Water extract from females	54	54	9 (16.7 %) ns	23 (42.6 %) ***	28 (51.9 %) ***	29 (53.7 %) ***	32 (59.3 %) ***
Water extract from females without gelatinous matrices	180	180	19 (10.6 %) ns	59 (32.8 %) ns	73 (40.6 %) ns	78 (43.3 %) ns	74 (41.1 %) ns
Water extract from gelatinous matrices	180	180	24 (13.3 %) ns	51 (28.3 %) ns	60 (33.3 %) ns	68 (37.8 %) ns	76 (42.2 %) ns

ns = no significant difference ($P \geq 0.05$); *** = $P < 0.001$; according to the Chi-square test with Yates correction.

sent in extracts from females with n-hexane, ethyl acetate, methylene chloride, methanol, and water (Table 1), indicating that these substances possess both polar and apolar properties. In contrast to these results, Clarke *et al.* (1976) found a high water solubility but insolubility in most common organic solvents for the sex pheromone of *G. rostochiensis*. A substance with sex pheromone activity from females of *H. glycines*, vanillic acid (Jaffe *et al.*, 1989), is soluble both in water and in alcohols and ether (Budavari, 1989) in concentrations that attracted males in the bioassay system used by these authors. Bone (1986) found two fractions of the *H. glycines* pheromone that were soluble in water and methanol, respectively.

According to Bone *et al.* (1980), the sex pheromone of *Nippostrongylus brasiliensis* is a long-chain polypeptide. Our findings (Table 1) indicate that the sex pheromonal substances from *H. schachtii* are not polypeptides because otherwise pronase and leucine aminopeptidase should have reduced their concentrations in the extracts from females. The observation that enzyme-treated extracts from females always attracted more males than extracts from females without enzymes (not statistically analyzed) may be explained by the finding of Huettel and Jaffe (1987) that males of *H. glycines* were attracted by several amino acids. In our experiments the enzymatic incubation of extracts from females may have liberated amino acids attracting *H. schachtii* males from female proteins that were solubilized during incubation. Furthermore the experiments show a remarkable temperature stability of the *H. schachtii* sex pheromonal substances. As mentioned above they remained attractive both after 20.5 h at 37 °C and after 18 days at 4-6 °C. These findings are supported by Green and Plumb (1970) who found that the sex attractants of several *Heterodera* species, including *H. schachtii*, remained attractive when stored for several months at 5 °C.

From interspecies experiments with cyst nematodes Green and Plumb (1970) suggested that *H. glycines* and *H. schachtii* share common sex pheromone components. Jaffe *et al.* (1989) identified vanillic acid as a substance with sex pheromone activity for *H. glycines* males. The present study shows that vanillic acid in concentrations of 10 µmol/l, 1 µmol/l, and 100 nmol/l, was not attractive for *H. schachtii* males (Table 2). Vanillic acid may therefore not be a long range sex pheromone of *H. schachtii*.

According to Green and Greet (1972), the sex pheromone of *H. schachtii* is released all over the body with a higher portion in the posterior than in the anterior region. From the data in this study (Table 2), the gelatinous matrix released through the vulval opening obviously acts as an attractant carrier. The apolar nature of the sex pheromonal substances (Table 1) does not argue against a release through the cuticle but the high attractivity of the gelatinous matrix extract and its relatively small size compared to the female body point to the vulval opening as the main site of release.

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