Pheromones of the housefly

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Chapter 5

'SELF-LOADING' WITH SEMIOCHEMICALS BY HOUSEFLIES (*MUSCA DOMESTICA* L.): A NEW TECHNIQUE

Abstract

It was shown that application of cuticular semiochemicals (long-chain hydrocarbons) dissolved in hexane or acetone to the thorax of houseflies (*Musca domestica* L.) may strongly affect the flies' condition. In addition, the distribution of these substances over the various body parts may deviate from the natural distribution. It appeared that hydrocarbons, when liquid at room temperature, are also taken up and that they are distributed over the body in a more natural way when the flies are walking on a filter paper onto which the pure chemicals had been pipetted. The higher the amounts of hydrocarbons on the paper and the longer the flies walked on it, the higher the amounts of chemicals taken up.

Using this new "self-loading" technique, females were loaded with (Z)-9-heptacosene or (Z)-9-pentacosene. The former substance is the most abundant hydrocarbon on the cuticle of male houseflies and hardly occurs on females. (Z)-9-pentacosene is absent on houseflies, but acts as a female sex pheromone of the little housefly *Fannia canicularis*, which often is sympatric with *M. domestica*. We hypothesize that both (Z)-9-heptacosene and (Z)-9-pentacosene may inhibit sexual behaviour in male houseflies. However, we now show that in contrast to this (Z)-9-heptacosene stimulates copulation when present in relatively high amounts on females, whereas (Z)-9-pentacosene did not affect male sexual behaviour.

Introduction

Studies to reveal the role of cuticular semiochemicals on sexual behaviour of *Musca domestica* often imply topical application of these chemicals on the flies. The chemicals are usually dissolved in an organic solvent like hexane, acetone or petrol-ether after which a few microliters of the solution are pipetted onto the dorsal part of the fly. In this way the amount of semiochemical applied is exactly known (Adams *et al.*, 1987; La France, 1989; Islam and Port, 1994). This technique, however, has a number of disadvantages. The aggressive solvents may disturb the natural chemical matrix of hydrocarbons present on the cuticle or may partly disrupt the cuticle itself (Beament, 1945). In addition, the distribution of the applied chemicals over the body parts may differ from the natural distribution of these chemicals. These effects are very likely to influence the normal behaviour of the flies.

In the present study we investigated possibilities to apply semiochemicals in a non-aggressive, more natural way and, as a result, to obtain a more natural starting point for behavioural studies. We compared the distribution of hydrocarbons on untreated female flies with the distribution of these substances after they had been taken up from a filter paper on which the females had been walking or after these substances had been applied topically in a solvent.

(Z)-9-Heptacosene is the most abundant hydrocarbon present on the cuticle of male flies. Unmated females have this substance only in small amounts. Rao *et al.* (1988, 1990) stated that (Z)-9-heptacosene is a part of the male sex pheromone of *M. domestica* and is attractive to female houseflies. Schlein *et al.* (1981) found that hexane-soluble substances on the cuticle of male flies terminate courting by other males on contact. The inhibitory activity of these “abstinons” follows from the fact that males did not mate with females treated with male extract. We hypothesized that (Z)-9-heptacosene may repel males, thereby preventing homosexual contacts between them. As a consequence, a relatively high amount of (Z)-9-heptacosene on females might have an inhibitory effect on mating behaviour of male flies.

The cuticular (Z)-9-alkenes produced by female houseflies all possess carbon chains with an odd number of C atoms ranging from 21 to more than 31. In this range only (Z)-9-pentacosene is absent (Nelson *et al.*, 1981). Mansingh *et al.* (1972) found weak behavioural responses of male flies to (Z)-9-pentacosene in olfactometer tests when compared to those to (Z)-9-tricosene, (Z)-9-tetracosene and a 7:3 mixture of (Z)-9-heneicosene and (Z)-9-tricosene. (Z)-9-pentacosene constitutes 66% of the cuticular lipids of females of the little housefly *Fannia canicularis* and induces copulatory responses in males of this species towards pseudoflies treated with 100 or 200 µg of this substance (Uebel *et al.*, 1977). Other mono-olefins, including (Z)-9-heptacosene and (Z)-9-tricosene, do not elicit responses in male *F. canicularis*. Therefore, the authors concluded that (Z)-9-pentacosene is used by male *F. canicularis* as a species- and sex-recognition pheromone. Since the habitats of *M. domestica* and *F. canicularis* overlap, this led us to assume that (Z)-9-pentacosene may play a role in reproductive isolation of these two species and may have an inhibitory effect on courtship behaviour of male *M. domestica.* To investigate this, we studied courtship behaviour of male houseflies towards females which had taken up (Z)-9-heptacosene or (Z)-9-pentacosene from a filter paper.

Materials and methods

Insects

Experiments were done with *Musca domestica* L. flies from two different strains: A laboratory strain (WHO Ij2) which was obtained from the Statens Skadedyrlaboratorium, Lyngby (Denmark), and a wild-type strain obtained from a cow-house with pig-sty (Pesse strain) in The Netherlands. Flies of the WHO strain were in culture since 1961, flies of the Pesse strain had been reared in the laboratory for about 2 years. The WHO females contained relatively high amounts of (Z)-9-tricosene (approx. 10 µg/fly), whereas the Pesse females produced relatively low amounts of this substance (approx. 1 µg/fly). Depending on the objectives of the experiments either WHO flies or Pesse flies were used. The flies were kept in cages (30 x 30 x 40 cm) in a L12 : D12 regime at 25 ^{o}C and r.h. 60%. They were fed a mixture of sugar, powdered milk and yeast (5 : 5 : 1 by weight). In addition, tap water was present. Experiments were done with flies, which were 4 or 8 days old.

Experiments

Survival test: Sixty female WHO flies, 4 days of age, were immobilized by low temperature (≈ 2 min at –5 ^{o}C). On 20 flies 1 µl hexane and on another 20 flies 1 µl acetone was pipetted onto the dorsal part of the thorax. On the remaining control group of 20 flies no chemical was applied. The three groups of flies were put in separate cages to recover. After 1 hour the condition of the females was determined.

Application of semiochemicals

Application and behavioural tests: The tested chemicals were applied on female flies in two ways: Topically on the thorax ((Z)-9-heneicosene) or by uptake from filter paper on which the flies were walking ((Z)-9-heneicosene, (Z)-9-tricosene, (Z)-9-pentacosene, (Z)-9-heptacosene and (Z)-9-nonacosene). Topical appliance was done by pipetting 1 µl hexane containing 10 µg of the test chemical onto the dorsal part of the thorax. Uptake of the semiochemicals from filter paper was established by putting the females in a Petri dish (9 cm diameter) the bottom of which was covered with filter paper. One or 5 µl of the pure chemical had been applied to the filter paper about 0.5 cm from the edge of the dish (flies prefer walking along the edge) at two opposite locations. The flies stayed in the dish for 30, 60 or 120 min and were then placed back in a cage. Within 1 to 5 hours after treatment the flies were used in behavioural experiments.

Behavioural experiments

The behaviour of 8-day-old Pesse flies was observed in Petri dishes (9 cm diameter). One virgin male and 1 virgin female were put in a dish through a hole in the centre of the cover after which the hole was closed. The numbers of copulation attempts (‘strikes’: Tobin and Stoffolano, 1973) and successful copulations were recorded during 10 minutes. After each experiment the dishes were cleaned in hot water containing detergent, rinsed in distilled water and then dried.

Chemical analyses

Cuticular hydrocarbons were determined on 8-day-old females. The flies were killed by freezing after which the head, legs, wings, thorax and abdomen were separated under a stereomicroscope using a forceps and scalpel. Each body part was separately immersed in 0.2 ml hexane, the whole was shaken during 1 min, after which the parts were kept in the fluid for at least 1 hour.

Gas chromatography was performed on a Hewlett Packard 5890 series II gas chromatograph. Two µl of the solution was injected into a WCOT fused-silica CP-Sil-5 CB column (25 m x 0.32 mm i.d., film thickness, 0.25 µm; Chrompack) with injector at 250 ^{o}C and FID at 300 ^{o}C. The flow rate of the nitrogen carrier gas was 26 cm/s. The split ratio was 56:1. GC oven temperature was programmed from 50 to 300 ^{o}C at 10 ^{o}C/min. 2-Nonanone was used as an internal standard. The most abundant hydrocarbons (alkanes and (Z)-9-alkenes) were identified by comparing their retention times with those of reference runs, with data from literature, and by mass spectrometry. No attempts were made to chemically identify the other hydrocarbons. The lower detection level of the individual hydrocarbons was in the order of 5 ng. Quantities of the hydrocarbons were expressed as micrograms.

Results

From the 20 flies treated with 1 µl hexane 45% died and only 20% fully recovered. 35 % of the flies did not fully recover, i.e., the flies were still alive but their mobility was very low or uncontrolled. From the 20 flies treated with 1 µl acetone 15% died and 80% fully recovered. All 20 untreated control flies fully recovered from chilling (Fig. 1).

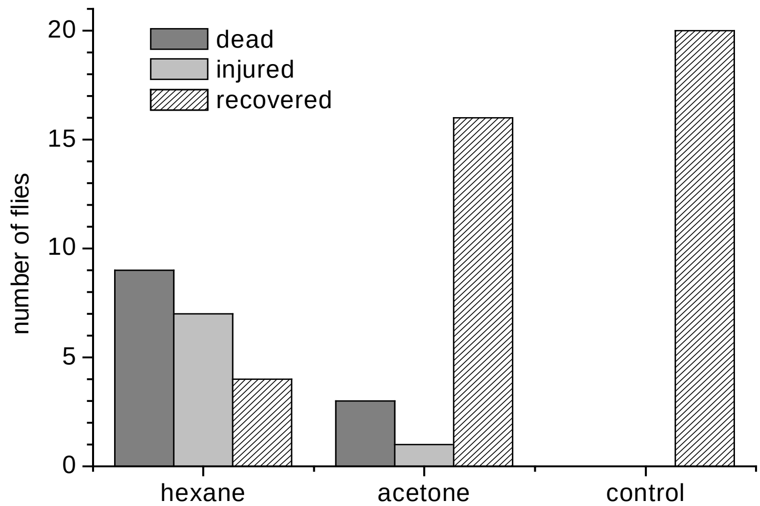

Figure 1. Condition of 4-day-old WHO female houseflies 1 hour after immobilization by low temperature (control), and after immobilization and subsequent treatment with 1 µl hexane or acetone. n=20 for each group.

The natural distribution of the most abundant alkanes and alkenes on the various body parts of 8-day-old WHO females is presented in Figure 2. Figure 2A shows the amounts in micrograms. It appears that on the head the total amount of the hydrocarbons was considerably lower than on the other parts of the body. In order to compare the hydrocarbon profiles on the various body parts (which differ considerably in size), the amounts of the various hydrocarbons are presented in percentages in Figure 2B. This figure shows that the hydrocarbon profiles were about the same for the legs, wings, thorax and abdomen. The distribution of the hydrocarbons on the head, however, differed from that on the other body parts. This difference was mainly caused by a shift in the alkane/alkene ratio. Figure 3 shows this difference in more detail. The ratio n-alkane/alkene for the various body parts did not differ for the legs, wings, thorax and abdomen. However, on the head this ratio was significantly higher (Mann Whitney U test, C23: $p < 0.05$, C27: $p < 0.001$, C29: $p < 0.001$).

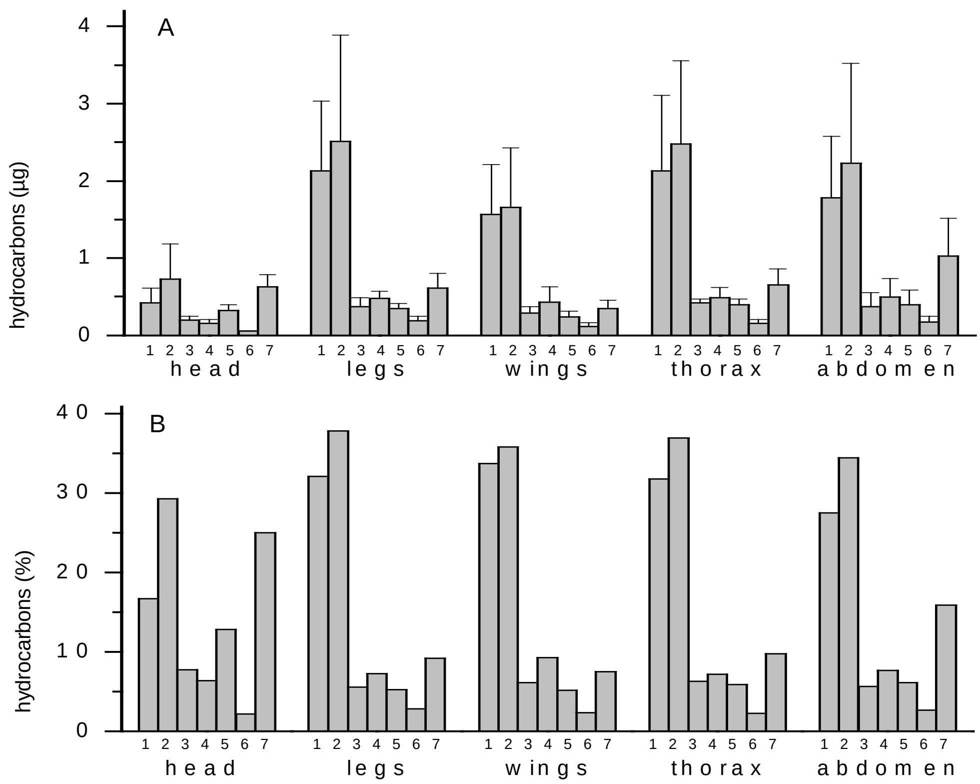

Figure 2. Natural distribution of the most abundant hydrocarbons on WHO females of *Musca domestica* (n=10). A: amounts, B: percentages on head, legs, wings, thorax and abdomen. 1=(Z)-9-tricosene, 2=tricosane, 3=pentacosane, 4=(Z)-9-heptacosene, 5=heptacosane, 6=(Z)-9-nonacosene and 7=nonacosane. Error bars denote standard deviations.

Figure 4 shows the amounts of (Z)-9-tricosene on Pesse females immediately after they had been removed from the rearing cage and on females of the same strain which had been walking, during 1 h, on filter paper onto which 2 drops had been pipetted of, 1 and 5 µl (Z)-9-tricosene, respectively. It is clear that during walking uptake of (Z)-9-tricosene had taken place and that the amounts taken up were higher the more of this substance was present on the filter paper. Compared to the control females an additional uptake of about 1 and 5 µg had occurred at doses of 1 and 5 µl, respectively.

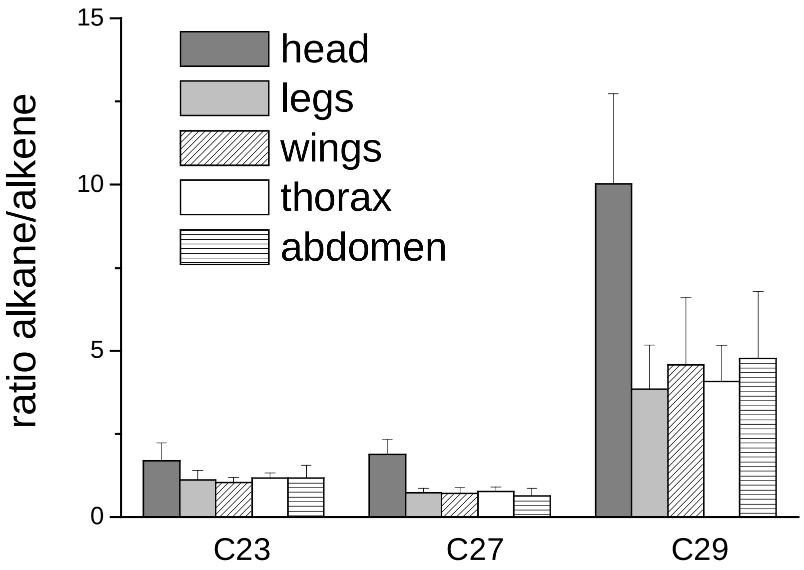

Figure 3. Ratio of alkanes and corresponding (Z)-9-alkenes on different body parts of female WHO *Musca domestica*. C23: tricosane/(Z)-9-tricosene, C27: heptacosane/(Z)-9-heptacosene, C29: nonacosane/(Z)-9-nonacosene (n=10). Error bars denote standard deviations.

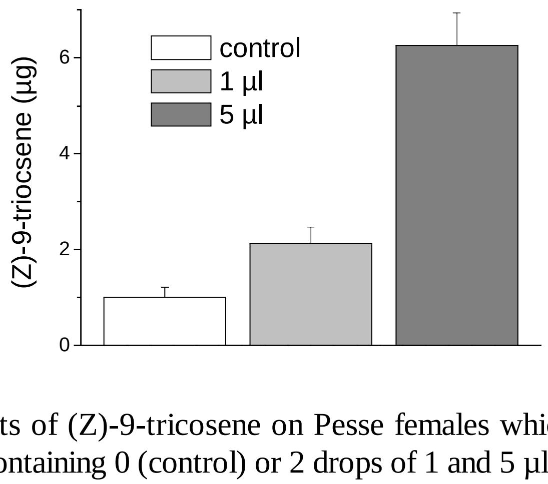

Figure 4. Total amounts of (Z)-9-tricosene on Pesse females which had been walking, during 1 hour on filter paper containing 0 (control) or 2 drops of 1 and 5 µl (Z)-9-tricosene, respectively. n=5 for each group. Error bars denote standard deviations.

Figure 5 shows the distribution on 8-day-old WHO females of (Z)-9-heneicosene when this substance had been applied directly to the thorax and when it had been taken up from filter paper. The natural distribution of (Z)-9-tricosene on 8-day-old WHO females served as a control. There was no significant difference in the distribution over the body parts between naturally occurring (Z)-9-tricosene and (Z)-9-heneicosene taken up from filter paper. However, when (Z)-9-heneicosene had been applied topically on the thorax significantly lower amounts of this substance were present on the legs and higher amounts on the wings compared with the amounts taken up from filter paper (Mann-Whitney U test, legs: $p < 0.001$, wings $p < 0.001$). These results indicate that in the latter case the semiochemicals were distributed over the body in a more natural way than when applied in a solvent to the thorax of the fly.

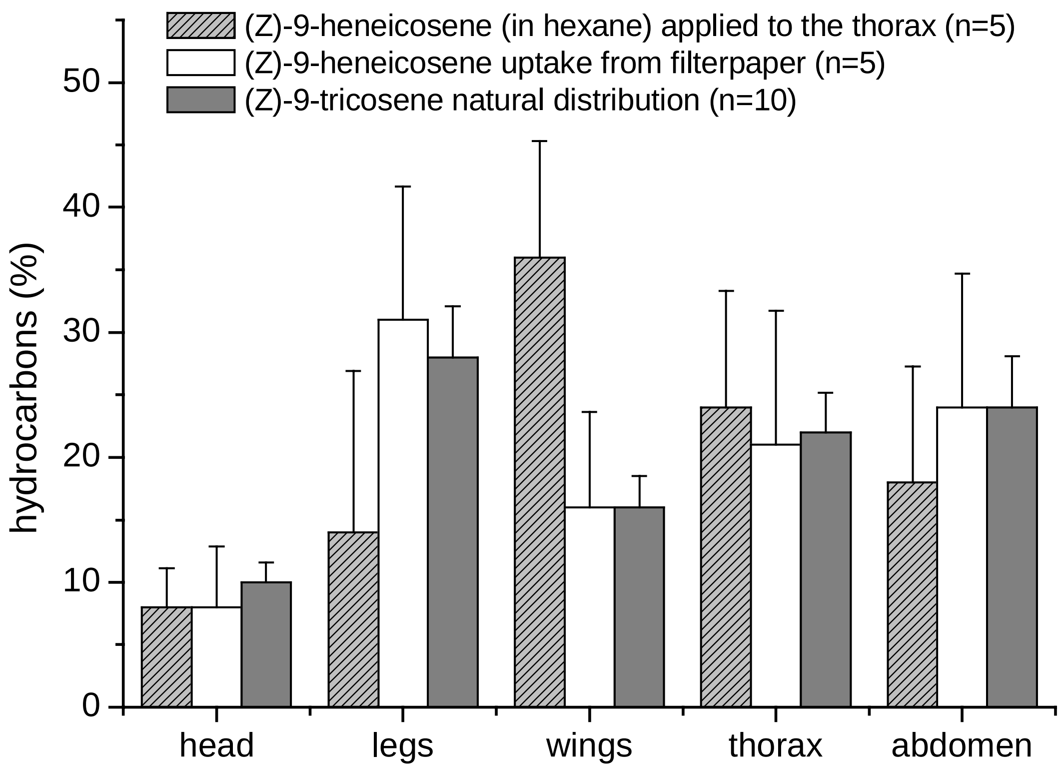

Figure 5. Distribution on 8-day-old WHO females of (Z)-9-heneicosene after topical application and after uptake from filter paper compared to the natural distribution of (Z)-9-tricosene. Error bars denote standard deviations.

In order to find out whether flies could also take up chemicals with high melting points from a filter paper, we tested the uptake of (Z)-9-heptacosene (liquid at room temperature) and (Z)-9-nonacosene (solid at room temperature). For this purpose 2 drops of 5 µl (Z)-9-heptacosene or (Z)-9-nonacosene (made liquid by heating) was pipetted onto filter paper. Figure 6 shows the amounts of (Z)-9-heptacosene and (Z)-9-nonacosene on 8-day-old WHO females which had been walking, for 1 h, on filter paper loaded with one of these substances. It appears that flies, which had been walking on filter paper with (Z)-9-heptacosene, had taken up an average of about 1 µg of this substance when compared to the amounts present on control flies. However, on flies which had been in contact with filter paper containing (Z)-9-nonacosene the amounts of this substance had not increased compared to the amounts of (Z)-9-nonacosene on the control flies.

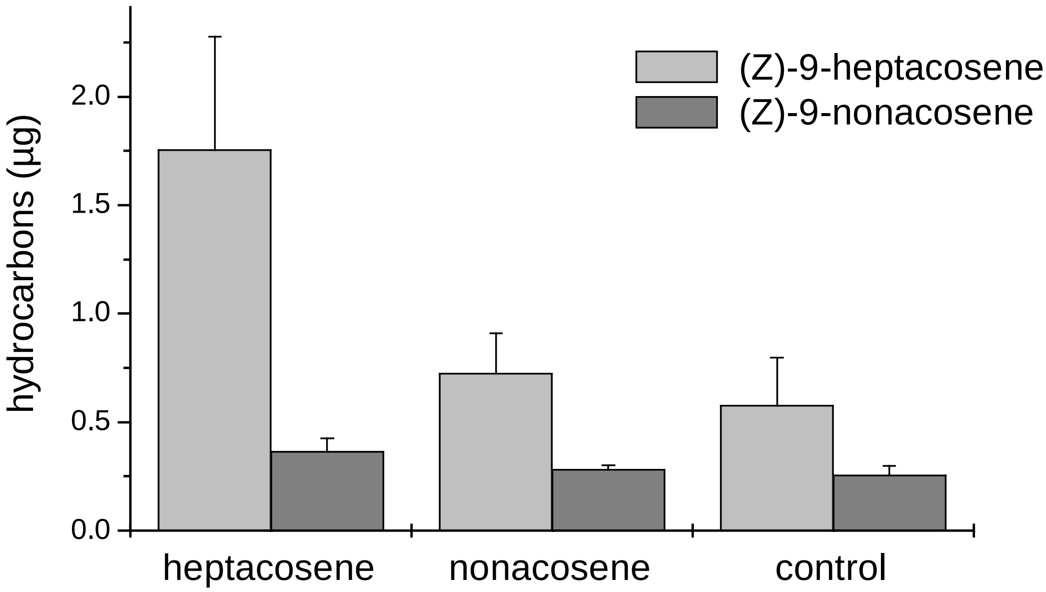

Figure 6. Amounts of (Z)-9-heptacosene and (Z)-9-nonacosene taken up by 8-day-old WHO female *Musca domestica* which had been walking, during 1 hour, on filter paper containing 2 drops of 5 µl of one of these hydrocarbons. Control flies had been walking on clean filter paper. n=10 for each group. Error bars indicate standard deviations.

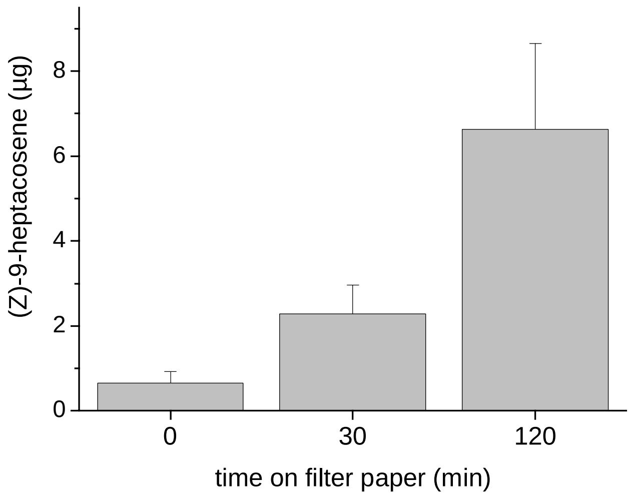

Figure 7. Amounts of (Z)-9-heptacosene present on 8-day-old virgin Pesse females which had been walking on filter paper loaded with 2 drops of (Z)-9-heptacosene during 0, 30 and 120 min. n=6 for each group. Error bars indicate standard deviations.

Figure 7 shows the amounts of (Z)-9-heptacosene are shown present on 8-day-old virgin Pesse females which had been walking on filter paper loaded with 2 drops of 5 µl of this substance for 0, 30 and 120 min, respectively. It is clear that the longer the flies had been walking on the paper the more (Z)-9-heptacosene had been taken up.

We carried out experiments in which each of the 6 females of each group was coupled with an 8-day-old virgin male in a Petri dish and observed if copulations occurred. It appeared that within 10-min periods no copulations took place when the females contained less than 1 µg (Z)-9-heptacosene. Two out of 6 males mated with females which had been on the filter paper for 30 min and contained 3.1 and 3.2 µg, respectively. All couples copulated when the females had been on the filter paper for 120 min; these females contained between 3.6 and 9.7µg (Z)-9-heptacosene. Hence, all eight females with which copulation had taken place contained more than 3 µg of (Z)-9-heptacosene.

We also investigated the sexual behaviour of Pesse males towards 10 Pesse females loaded with amounts of (Z)-9-pentacosene taken up from filter paper ranging from 0.3 to 10.1 µg/female. We found that copulation occurred with 3 females containing 0.4, 1.3 and 4.2 µg (Z)-9-pentacosene, respectively. From a control group of 10 Pesse females not containing (Z)-9-pentacosene 4 females copulated. It thus seems that the amount of (Z)-9-pentacosene on a female does not play a significant role in male sexual behaviour.

Discussion

Application of cuticular semiochemicals dissolved in rather aggressive solvents to female *Musca domestica* flies is a common method to study the effects of these substances on the behaviour of males. However, we showed that application of 1 µl hexane or acetone to the thorax of the females flies may strongly affect the condition of the insects. Only 20 % of the flies treated with hexane and 80 % of the flies treated with acetone fully recovered. The remaining flies died or lost control of mobility. The fact that acetone is less harmful than hexane may be due to the fact that the long-chain apolar cuticular hydrocarbons hardly or not dissolve in the more polar acetone.

Table 1. Percent distribution of (Z)-9-tricosene, (Z)-9,10-epoxytricosane (C23 epoxide) and (Z)-14-tricosen-10-one (C23 ketone) on different body parts of female laboratory-cultured *Musca domestica*.

	Noorman (2000) (Z)-9-tricosene	Dilwith (1982) (Z)-9-tricosene	Blomquist (1984) C23 epoxide	Blomquist (1984) C23 ketone
Head	10	8	13	7
Legs	28	31	33	38
Wings	16	15	13	14
Thorax	22	20	17	17
Abdomen	24	26	24	24

On determination of the natural distribution of various hydrocarbons over the body parts of the WHO flies we obtained results similar to those found on laboratory-cultured flies by other authors (Table 1). However, on application of these substances in a solvent to the flies' thorax the distribution of the chemicals over the various body parts deviates from the natural distribution, high amounts of the substances being present on the wings and only small amounts on the legs. In behavioural experiments this unnatural distribution may affect the flies' behaviour. Therefore we attempted the flies to take up cuticular hydrocarbons by having them walk on a filter paper onto which these substances had been pipetted. Indeed it appeared that in this -non-aggressive- way hydrocarbons were taken up and distributed over the flies' body in conformity with the distribution of naturally occurring hydrocarbons. The higher the amounts of hydrocarbons on the filter paper and the longer the flies walked on the paper, the higher the amounts of the chemicals taken up. Larger insects may need to be exposed for longer times to higher amounts of chemicals to be adequately loaded.

‘Self-loading’ of test chemicals can only be done with substances which are liquid at room temperature. (Z)-9-heptacosene, which is liquid at room temperature, was taken up from the filter paper, in contrast to (Z)-9-nonacosene, which at room temperature is still solid.

(Z)-9-heptacosene is the most abundant hydrocarbon present on the cuticle of male houseflies (about 10µg/fly). Females produce this substance in relative small amounts only (0.4 –1.0 µg/fly) (Nelson *et al.*, 1981; Noorman and Den Otter, 2001). The latter authors also showed that substantial amounts of (Z)-9-heptacosene are transferred from males to females (Chapter 1) due to physical contacts between the sexes. We hypothesized that mating behaviour of the males might be inhibited when high amounts of this substance would be present on the females. However, in contrast to this, (Z)-9-heptacosene appeared to stimulate copulation when present in relatively high (> 3 µg/fly) amounts on females and thus it seems not to indicate to males that females have mated before. According to Rao *et al.* (1988, 1990), (Z)-9-heptacosene is a part of the male sex pheromone of *M. domestica* and that it attracts the females. La-France *et al.* (1989) showed that mixtures of (Z)-9-alkenes (including (Z)-9-tricosene and (Z)-9-heptacosene) enhanced sexual activity in males and they suggested a synergistic effect of certain (Z)-9-alkenes.

Our hypothesis that (Z)-9-pentacosene might have an inhibitory effect on the sexual activity of male *M. domestica* clearly does not hold true. Adult females which had taken up (Z)-9-pentacosene from filter paper were not less or more attractive than females without (Z)-9-pentacosene. Probably (Z)-9-pentacosene plays no role in sex-recognition by *M.domestica.*

Finally, the results of this study indicate that the natural uptake of semiochemicals from filter paper can be a good alternative for the application of chemicals solved in rather aggressive oxidising solvents as, for instance, hexane. A more natural starting point for conducting behavioural studies is achieved by this new method.

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