Chapter 4

EFFECTS OF RELATIVE HUMIDITY, TEMPERATURE AND POPULATION DENSITY ON THE PRODUCTION OF CUTICULAR HYDROCARBONS IN THE HOUSEFLY *MUSCA DOMESTICA* L.

Abstract

The production of cuticular hydrocarbons by both males and females of *Musca domestica* after emergence under very wet conditions (90% r.h.) compared to the production at 50 and 20% r.h. is delayed up to at least 3 days. Eight days after emergence, however, males contain the same amounts of hydrocarbons at all three r.h. values, whereas females at 90% r.h. still possess less of these substances than at 50 and 20% r.h. It is suggested that this is due to the fact that males, being more active than females, need more cuticular hydrocarbons to prevent water-loss than females. No indication is found that the r.h. has a different effect on the production of muscalure by females than on the production of the other hydrocarbons.

Male and female flies produce more hydrocarbons at 35 °C than at 20 °C. On females, the relative amounts of nonacosane and methyl- and dimethylnonacosanes are significantly higher at 35 °C than at 20 °C.

Female flies produce some (Z)-9-tricosene after 8 generations at low population density in contrast to females at high population density which did not produce muscalure. We suggest that because of the relatively large contribution to the total population, the properties of a small number of females are likely to be expressed sooner in the next generations of small populations than in those of large populations.

Introduction

The cuticular hydrocarbons of insects provide a barrier to water diffusion, thus preventing desiccation of the animals. However, some of these substances may also act as semiochemicals and may play a role in mating behaviour. In houseflies, several hydrocarbons on the body of females induce sexual behaviour in males. (Z)-9-tricosene is considered to be the main component of this sex pheromone (Carlson *et al.*, 1971, 1974; Mansingh *et al.*, 1972; Richter *et al.*, 1976; Uebel *et al.*, 1976; Rogoff *et al.* 1980; La-France *et al.*, 1989; Adams *et al.*, 1995).

It is known that environmental factors may affect the production of cuticular hydrocarbons (Hadley, 1977, 1978; Toolson *et al.*, 1990; Gibbs *et al.*, 1991). In several species of arthropods it has been found that at higher temperatures and lower humidities the proportion of cuticular n-alkanes is higher than that of branched alkanes and/or unsaturated hydrocarbons. These differences in hydrocarbon composition account for concomitant differences in cuticular permeability to water (Toolson and Hadley, 1979; Hadley and Schultz, 1987). Gibbs *et al.* (1995) suggested that production of sex pheromone by *Musca domestica* females may increase cuticular transpiration. Because the pheromone components are unsaturated or methyl-branched this might lead to higher permeability to water.

In previous studies (Noorman and Den Otter, 2001) we found that (Z)-9-tricosene comprised up to 20-30 % of the total hydrocarbons on females of a strain that had been cultured for many generations in the laboratory at constant temperature and humidity. On wild-type females, however, less than 0.5 % of this component was present. It appeared that on females of subsequent generations of laboratory populations the quantities of both (Z)-9-tricosene and tricosane –the production processes of which were shown to be closely linked- increased, whereas the amounts of the other cuticular hydrocarbons remained the same. This led us to assume that, although temperature and humidity may affect the production of all cuticular hydrocarbons, temperature and humidity mainly determine the quantities of the non-pheromonal components. (Z)-9-tricosene, however, may primarily be produced as a result of a selection process in high-density populations in isolated environments, females with higher quantities of this substance on their body being more attractive to males. These females may be the first to mate and oviposit fertile eggs. Their progeny may therefore survive in higher numbers in the next generation than

that of females producing lower amounts of (Z)-9-tricosene. It may be expected that this selection proceeds faster in high-density than in low-density populations.

This paper presents results of studies designed to test these hypotheses. We investigated the effects of relative humidity, temperature and population density on the production of various hydrocarbons in males and females of *Musca domestica*.

Materials and Methods

Insects

Experiments were carried out with *Musca dom*estica L. the larvae of which had been obtained from a cow-house with pig-sty (Pesse strain) and a poultry breeding (Van Diermen strain) in the Netherlands, respectively.

To investigate the effects of relative humidity on cuticular hydrocarbon production experiments were carried out with Pesse flies cultured in the laboratory for about 40 generations (Pesse-40) at 25 °C, r.h. 50 % and L12 : D12. Eight-day-old females of this strain contained about 3 μg muscalure/fly.

Immediately after emergence 10 male and 10 female flies were placed together in a cage (15 x 8 x 5 cm). Six cages were used, 2 of which were kept at about 90 % r.h., 2 at 50 % r.h. and 2 at about 20 % r.h. In order to obtain 90 % r.h., the cages were placed on a rack in the rearing room in a Perspex container (50 x 50 x 50 cm) the bottom of which was covered with a layer of tap water. Ventilation took place through holes in the walls of the container. To achieve 20 % r.h. the bottom of the container was covered with oven-dried silicagel, which was replaced before it was saturated with water. For 50 % r.h. the cages were placed in an empty container of which the upper cover was removed. Every day the actual temperature and r.h. in the cages were recorded. The temperature in the cages appeared to be 25.8 ± 0.5 °C, and r.h. was 19.1 ± 1.5 %, 47.2± 1.4 % and 91.8 ± 2.0 % respectively. Hydrocarbon quantities were determined on flies of each of these groups 3 and 8 days after emergence.

To establish the effects of temperature, Pesse flies were used from a strain reared for about 30 generations (Pesse-30) in the laboratory at 25 °C, r.h. 50 % and L12 : D12. Eight-day-old females of this strain contained about 1 μg muscalure/fly. Hydrocarbon quantities of 8-day-old flies from two cultures were compared. Immediately after

emergence, the flies of these cultures had been placed at 20 and 35 °C, respectively, in cages (30 x 30 x 40 cm) containing about 100 males and females.

The effects of population density on cuticular hydrocarbon composition were studied using Van Diermen flies. Flies originating from the 1st generation grown in the laboratory were reared for 8 successive generations (F1-F8) at 25 °C, r.h. 50 % and L12 : D12 in cages (30 x 30 x 40 cm) containing either less than 20 or more than 300 flies. Hydrocarbon quantities on 8-day-old flies of the F1 and F8 generations were compared.

All flies were fed a mixture of powdered milk, sugar and yeast (5 : 5 : 1 by weight). Water was present ad libitum in a tube filled with cotton.

In the first 2 series of experiments flies of only 1 generation, that already produced considerable amounts of muscalure were studied, because we expected an immediate effect on cuticular hydrocarbon production when changing the abiotic factors temperature and relative humidity. Since, however, the amounts of (Z)-9-tricosene production is supposed to be primarily the result of a selection process in isolated high-density populations, we compared in the third series flies from two generations, starting with a culture that did not produce detectable amounts of (Z)-9-tricosene.

Chemical analysis

Individual flies were immersed in 0.2 ml hexane, the whole was shaken during 1 min, after which the fly was kept in this fluid for 1 hour. Gas chromatography was performed on a Shimadzu GC-17A. 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. Muscalure and the other hydrocarbons were identified by comparing the retention time with reference runs of alkanes and (Z)-9-alkenes, or with data from the literature. Quantities of the hydrocarbons were expressed as percentages of the internal standard or as micrograms.

Results

Figure 1 shows the total amounts of cuticular hydrocarbons on males and females, 3 and 8 days old, which had been kept at different relative humidities from emergence. It appears that on both 3-day-old males and females the amounts of hydrocarbons were higher at 20 and 50 % r.h. than on those kept at 90 % r.h. (Mann-Whitney U test, $p < 0.01$). Eight days after emergence the total hydrocarbon quantities on the flies had become significantly higher at all r.h. levels compared to day 3 (Mann-Whitney U test, $p < 0.01$). In addition, the amounts on 8-day-old males at 90% r.h. were no longer lower than on those kept at 50 and 20 % r.h. This is in contrast to the females of which 8-day-old specimens at 90 % r.h. still contained less hydrocarbons than those kept at the lower r.h.'s (Mann-Whitney U test, $p < 0,05$).

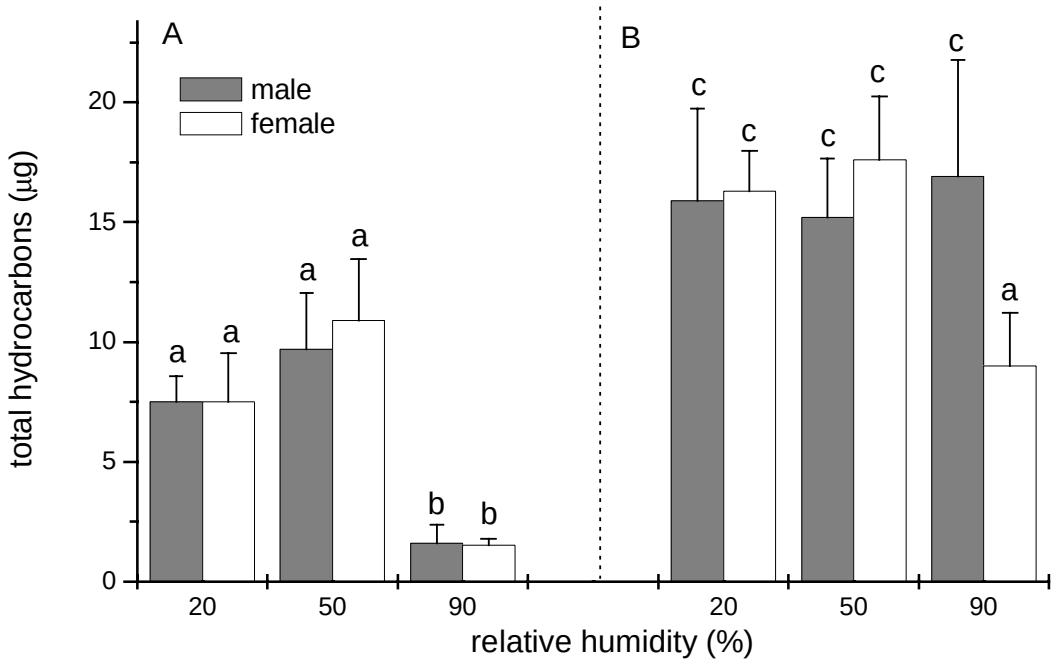

Figure 1. Amounts of total cuticular hydrocarbons of males (n=5) and females (n=10) of *M. domestica*, 3 (A) and 8 (B) days after emergence, at different relative humidities. Error bars represent standard deviations. Averages labeled with different letters differ significantly.

Figure 2 shows the amount of the most abundant hydrocarbon molecules present on the cuticle of the flies. These molecules cover about 90 % of the total cuticular hydrocarbons. The hydrocarbon 'profiles' of males and females differed considerably, males having relatively high quantities of (Z)-9-heptacosene, whereas on females relatively high amounts of (Z)-9-tricosene, tricosane and (Z)-9-heptacosene were present. On day 8 the amounts of hydrocarbons were higher than on day 3, but the hydrocarbon profiles of the males and females were still about the same. The amounts of (Z)-9-tricosene plus tricosane on females are about 15% of the total cuticular hydrocarbon quantity on day 3 after emergence and about 30% of the total on day 8 at each of the three r.h.'s.

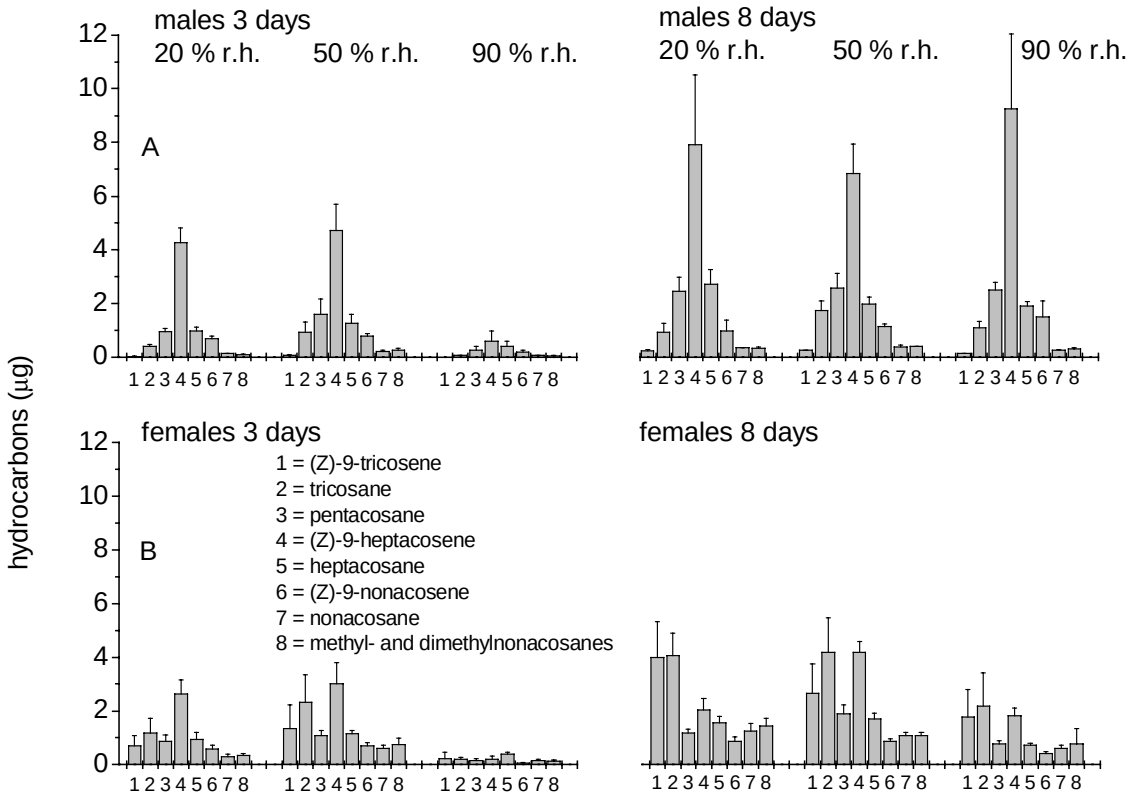

Figure 2. Amounts of cuticular hydrocarbons on males (A, n=5)) and females (B, n=10) of *M. domestica*, 3 and 8 days after emergence, at different relative humidities. Error bars denote standard deviations.

Figure 3 shows the hydrocarbons present on 8-day-old male and female flies at different temperatures. Striking differences in the total amounts of hydrocarbons were present between flies kept at 20 °C and at 35 °C. Males at 35 °C produced about 80 % more hydrocarbons than males kept at 20 °C (19.5 and 10.7 µg, respectively); females at 35 °C produced about 50 % more than females kept at 20 °C (21.2 and 13.9 µg, respectively). In male flies the relative amounts of the various hydrocarbons produced at the two temperatures did not differ significantly, whereas in female flies the relative amounts of nonacosane, and of the methyl- and dimethylnonacosanes were significantly higher at 35 °C than at 20 °C (t-test, $P<0.01$). Although at 20 °C females produced less muscalure than at 35 °C (1.0 vs. 1.4 µg) this difference was not significant. The relative amounts of the remaining hydrocarbons of females kept at 20 °C and at 35 °C did not differ. Hence, the production of muscalure is not deviant from the production of these hydrocarbons at the two temperatures.

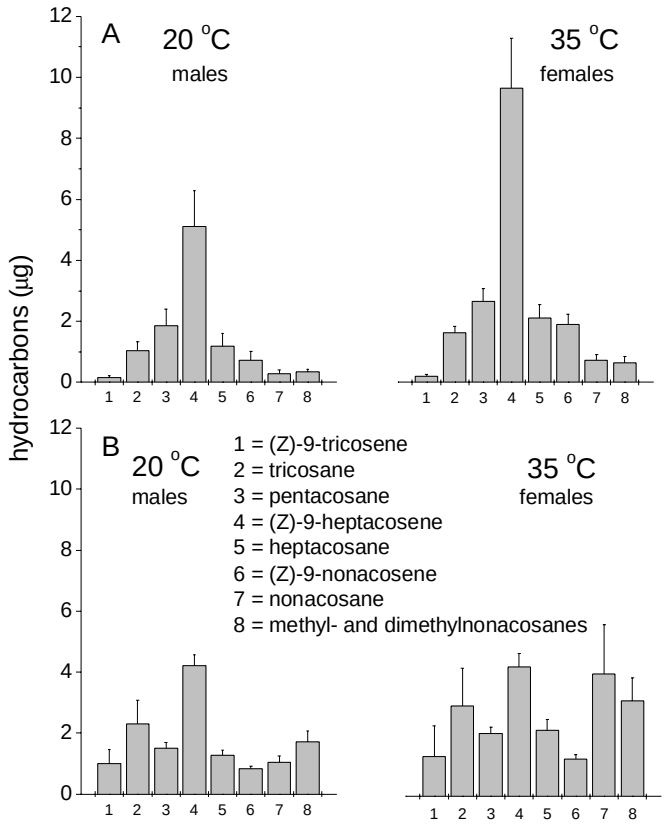

Figure 3. Amounts of cuticular hydrocarbons on 8-day-old males (A, n=5) and females (B, n=10) of *M. domestica* at 20 and 35 °C. Error bars denote standard deviations.

Figure 4 shows the results of the selection experiment with Van Diermen flies.

The total amounts of the hydrocarbons on the first generation males (11.9 µg) and females (16.5 µg) did not differ significantly from those on the 8th generation flies kept at low or high density (males 11.4 and 14.3 µg, females 19.2 and 14.0 µg, respectively (Mann-Whitney U test, $p > 0.05$). The hydrocarbon profiles of the males were similar to those of the males of the Pesse strains used in the previous experiments (cf. Figs. 2A , 3A and 4A), whereas those of the females differed considerably from the Pesse females (cf. Figs. 2B, 3B and 4B). On 8-day-old Van Diermen females, nonacosane and methyl- and dimethylnonacosanes were the most abundant hydrocarbons. Females of the low-density population contained (Z)-9-tricosene, although the amounts were low.

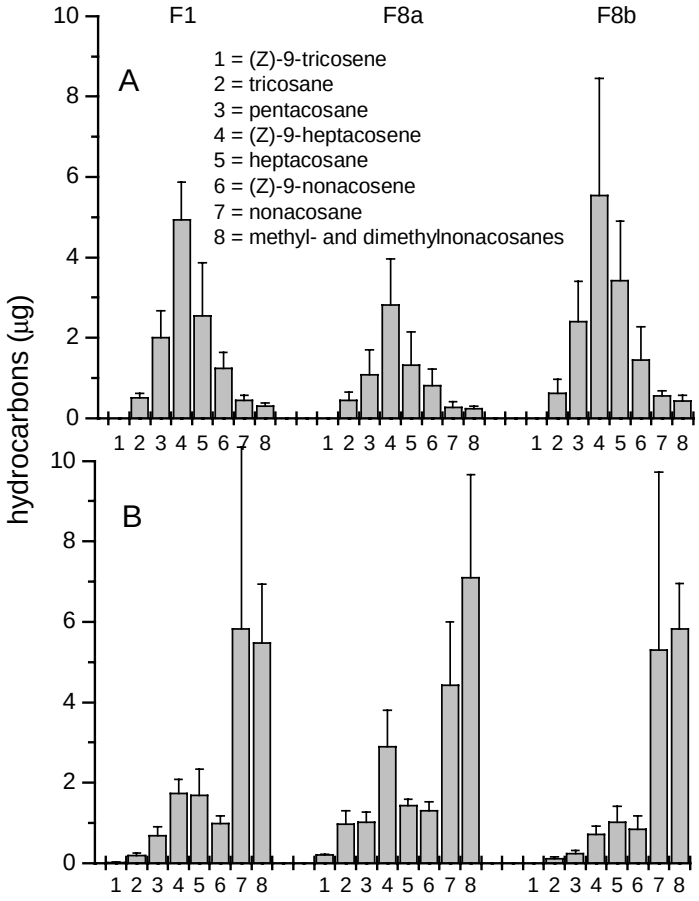

Figure 4. Amounts of cuticular hydrocarbons on 8-day-old 1st laboratory generation (F1) males (A) and females (B) of *M. domestica* and on males and females of the same strain kept in culture for 8 generations at different population densities (F8a <20 and F8b >300 flies/cage). n=10 for each generation. Error bars indicate standard deviations.

Discussion

Evaporation of water through the cuticle can be expected to be lower at higher r.h. Therefore, fewer cuticular hydrocarbons may be needed to protect the flies from desiccation. Toolson and Hadley (1979) observed marked seasonal changes in the relative abundance of cuticular hydrocarbons of the scorpion *Centruroides sculpturatus,* the percentage of *n*-alkanes in the epicuticular hydrocarbon decreasing considerably in warmer months, whereas the proportion of branched alkanes increased. Hadley and Schultz (1987) showed a correlation between water loss rates and quantities and composition of cuticular hydrocarbons. In the tiger beetle *Cicindela obsoleta*, a summer-active species that inhabits dry grasslands, the cuticular hydrocarbon fraction only contains saturated hydrocarbons, whereas on the body of *C. oregona*, a spring- and fall-active species which lives along water courses, about 50% of the hydrocarbons are unsaturated. The presence of unsaturated hydrocarbons is thought to increase the permeability of the hydrocarbon layer to water. Indeed, Hadley and Schultz (1987) found a significant negative correlation between water loss rate and the quantity of saturated hydrocarbons.

We found that both males and females of 3 days old reared at 90 % r.h. possessed remarkably lower amounts of saturated as well as unsaturated hydrocarbons than those reared at 20 and 50% r.h. Eight days after emergence, however, males kept at 90% r.h. contained the same amounts of hydrocarbons as those kept at 20 and 50% r.h., whereas females still had less hydrocarbons on their cuticle. A possible explanation may be that, in general, males are more active than females and as a consequence may need more cuticular hydrocarbons to prevent water-loss than the less active females. The hydrocarbon profiles of the females cultured at different r.h. are about the same. This strongly suggests that our hypothesis that the production of (Z)-9-tricosene, the main component of the female sex pheromone, may not be affected by changes in humidity to the same extent as that of the other hydrocarbons does not hold true.

The melting temperature of the alkenes, methylalkanes and alkanes isolated from 4-day-old houseflies are respectively –0.2, 29.9 and 41.5 °C (Gibbs *et al.*, 1995). The melting temperature (midpoint of phase transition) of the mixture of all cuticular hydrocarbons was found to be 36.8 °C in females and 39.4 °C in males. However, the transition of lipids from the solid to the liquid phase ranges over 10-15 °C. The necessity to keep the melting temperature of the cuticular lipid-mixture within a certain range can

probably affect the amounts in which certain hydrocarbons are produced, depending on different environmental circumstances. We observed that the relative amounts of nonacosane and methylnonacosanes on females were higher at 35 °C than at 20 °C. This may have led to an increase of the melting temperature of the whole mixture of cuticular hydrocarbons. The hydrocarbon profiles on male flies were, however, the same at the two temperatures. The melting temperature of all cuticular hydrocarbons is higher in males than in females (Gibbs *et al.*, 1995). Probably there is no reason to change the amount and composition of hydrocarbons on males in an environmental temperature range of 20 °C to 35 °C.

In the low-density population cultures (<20 flies/cage) all females of the 8th generation produced a low amount of muscalure, whereas no muscalure was found on the high-density (>300 flies/cage) females. We were enable to compare 8 generation but, nevertheless, after this limited number of generations differences in (Z)-9-tricosene production between the two populations could already be observed. We found (Z) 9 tricosene on all the females of the analysed low-density population where each fly contained about the same amount of this substance. Surprisingly, none of the females of the high-density populations produced detectable amounts of this substance. Probably this difference developed by chance. The properties of one or a few females are likely to be expressed in relatively more individuals in the next generations of small (laboratory culture populations) than in large (wild-type) populations. It is clearly shown that selection can sneak in very rapidly. This effect should always be a point of major attention in laboratory colonies.

The clear difference in the hydrocarbon profiles between 8-day-old females of the Pesse-30 and -40 strains and the Van Diermen strain may be explained by focussing on the main function of cuticular lipids in general, i.e. to provide a barrier to water loss. The insects will need a certain amount of cuticular lipids in order to regulate the water-balance in an effective way. When (Z)-9-tricosene and tricosane in female houseflies are only present in small amounts, this paucity is probably compensated for by other hydrocarbons (nonacosane and methyl- and dimethylnonacosanes). This assumption seems to be plausible when considering the total amount of hydrocarbons on females of the Pesse-40 and Van Diermen strains in the experiments carried out at the same temperature and humidity (25 °C, r.h. 50%). Females of both populations produced about the same amount of total hydrocarbons irrespective of the hydrocarbon profiles. In

previous studies Noorman and Den Otter (2001) also found that both first-generation Van Diermen and Pesse females in the laboratory produced very low or no detectable amounts of (Z)-9-tricosene and relatively high amounts of nonacosane and methyl- and dimethylnonacosanes. Since no significant differences in the hydrocarbon profiles between these first-generation females of the Pesse and Van Diermen strains existed, strain differences do not appear to be responsible for the differences in hydrocarbon profiles.

The overall conclusions of the experiments are that relative humidity and temperature have a prominent effect on the production of cuticular hydrocarbons and that the effects on the production of muscalure and the other hydrocarbons are similar. The population-density experiment showed that in a small number of generations selection may give rise to differences in muscalure production between populations. The fact that in contrast to high-density populations females of the low-density population cultures produced some muscalure suggests that population density is not the main factor for inducing muscalure production.

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