

BEYOND 9-ODA: SEX PHEROMONE COMMUNICATION IN THE EUROPEAN HONEY BEE *Apis mellifera* L.

AXEL BROCKMANN,^{1,2,*} DANIEL DIETZ,¹ JOHANNES SPAETHE,^{1,3}
and JÜRGEN TAUTZ¹

¹*Beegroup Würzburg, Department of Behavioral Physiology and Sociobiology,
University of Würzburg, Germany*

(Received June 29, 2005; revised September 18, 2005; accepted October 28, 2005)

Published Online April 4, 2006

Abstract—The major component of the mandibular gland secretion of queen honeybees (*Apis mellifera* L.), 9-ODA ((2*E*)-9-oxodecenoic acid), has been known for more than 40 yr to function as a long-range sex pheromone, attracting drones at congregation areas and drone flyways. Tests of other mandibular gland components failed to demonstrate attraction. It remained unclear whether these components served any function in mating behavior. We performed dual-choice experiments, using a rotating drone carousel, to test the attractiveness of 9-ODA compared to mixtures of 9-ODA with three other most abundant components in virgin queen mandibular gland secretions: (2*E*)-9-hydroxydecenoic acid (9-HDA), (2*E*)-10-hydroxydecenoic acid (10-HDA), and *p*-hydroxybenzoate (HOB). We found no differences in the number of drones attracted to 9-ODA or the respective mixtures over a distance. However, adding 9-HDA and 10-HDA, or 9-HDA, 10-HDA, and HOB to 9-ODA increased the number of drones making contact with the baited dummy. On the basis of these results, we suggest that at least 9-HDA and 10-HDA are additional components of the sex pheromone blend of *A. mellifera*.

Key Words—Sex pheromone, mating behavior, mandibular gland, honeybee, *Apis mellifera*, (2*E*)-9-oxodecenoic acid, (2*E*)-9-hydroxydecenoic acid, (2*E*)-10-hydroxydecenoic acid.

* To whom correspondence should be addressed. E-mail: abrockma@life.uiuc.edu

² Current address: Department of Entomology, Neuroscience Program, University of Illinois, Urbana-Champaign, Urbana, Illinois, USA.

³ Current address: Department of Evolutionary Biology, University of Vienna, Vienna, Austria.

INTRODUCTION

In insects, the most elaborate chemical communication systems have evolved in the context of mating and social behaviors (Wilson, 1971; Phelan, 1992; Ayasse et al., 2001). In honeybees, these communication systems are closely linked because the virgin queen uses the mandibular gland secretions to attract drones on her mating flights (Gary, 1962; Butler et al., 1967), whereas the mated queen uses mandibular gland secretions to signal her presence to workers in the hive (Velthuis, 1985; Free, 1987; Slessor et al., 1988; Brockmann et al., 1998). The complex and phase-dependent (i.e., virgin vs. mated) chemical compositions of queen mandibular gland secretions (Plettner et al., 1997; Free, 1987) suggest that single components or mixtures of components are addressed differentially to these two receiver groups and may serve different functions in specific behavioral contexts.

In the 1970s, (2*E*)-9-oxodecenoic acid (9-ODA), the major component of the mandibular gland secretions, was shown to attract drones to queen dummies (Butler, 1971; Gary and Marston, 1971; Boch et al., 1975; Free, 1987). Further investigations suggested that other components of the secretion might play a role in the communication between sexes (for a detailed discussion of these experiments, see Free, 1987; Koeniger and Koeniger, 2000). More recent experiments, which tested the attractiveness of the six most abundant mandibular gland components: 9-ODA, (2*E*)-9-hydroxydecenoic acid (9-HDA; 85% (*R*)-(-), 15% (*S*-(+)), (2*E*)-10-hydroxydecenoic acid (10-HDA), *p*-hydroxybenzoate (HOB), and 4-hydroxy-3-methoxyphenylethanol (HVA), demonstrated that 9-ODA—but none of the other components—attracted drones from a distance (Loper et al., 1996). However, the inability of a single component to attract drones does not necessarily exclude a pheromonal function of a particular component as part of a blend (Linn et al., 1986; Christensen, 1997). Thus, it was not clear whether honeybee sex pheromone communication was based on a single substance or a multicomponent blend, and if the latter, which of the other mandibular gland compounds are also sex pheromone components. In contrast, in the communication between queens and workers, it has been shown that a blend of five mandibular gland substances is necessary to elicit worker retinue behavior [9-ODA, 9-HDA (85% (*R*)-(-), 15% (*S*-(+)), HOB, and HVA] (Slessor et al., 1988; Plettner et al., 1996, 1997).

In the experiments reported here, the potential sex pheromonal function of mandibular gland components were assessed in dual choice experiments using a motor-driven drone carousel, by testing attractiveness of 9-ODA vs. different combinations of 9-ODA and minor mandibular gland components.

METHODS AND MATERIALS

Experimental Site. Experiments were conducted in the vicinity of a drone congregation area close to the bee station and apiary of the Beegroup Würzburg

from May to July 2002. The experimental site was identified by presenting queen dummies containing $70.4 \mu\text{g}$ 9-ODA (equals the amount of 9-ODA in virgin queens' glands; Plettner et al., 1997) on the tip of a 3 m long rod at different spots within an area of about 0.3 km^2 between the bee station and the Biocenter of the University of Würzburg, Germany. The drone carousel was placed in an open meadow near a tree line at the edge of an orchard (marked by an "x" in Figure 1). No drones were detectable at the experimental site when no pheromones were presented, but when we offered a pheromone bait, drones

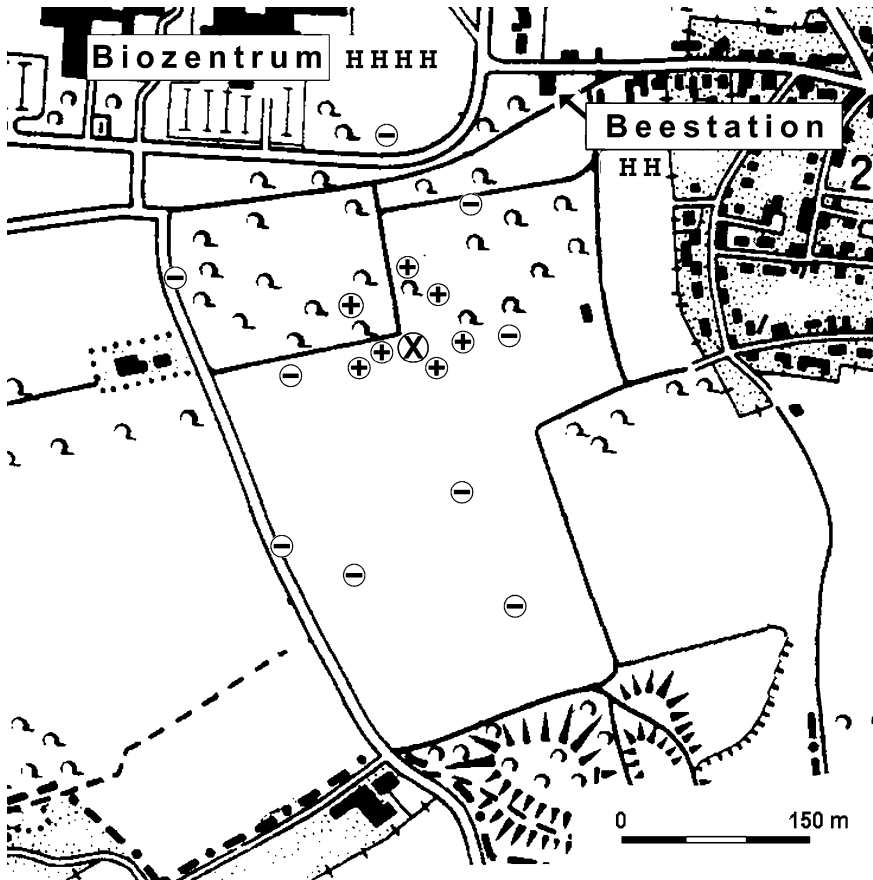


FIG. 1. General map of experimental site. (+) indicates sites at which drones could be attracted; (-) indicates sites at which drones could not be attracted by an artificial pheromone lure attached to a rod (3 m high), containing 1 Qeq of 9-ODA. (x) Position of the drone carousel; (H) position of closest bee hives.

appeared immediately. In some cases, we observed drones coming directly out of the trees lining the field. Thus, we assume that they were either cruising close to or were waiting on the trees. Prior to the experiments, we conducted a dose-response test to determine a pheromone dose that attracted an appropriate number of drones that still could be resolved on videotapes. Despite the use of standardized pheromone doses, drone numbers varied from day to day and between experiments on a single day.

Two-armed Drone Carousel. The drone carousel was a modification of that developed by Renner and Vierling (1977) and further improved by Gries and Koeniger (1996). It was composed of an upright column (adjustable for height from 4 to 6 m) carrying two opposing horizontal aluminum rods (each 2 m in length). The arms could be rotated with an electric motor around the vertical axis with a velocity of 3.1 m/sec at the tips of the arms. The queen dummies were fixed at the distal tips of the arms using a thin nylon thread (10 cm; height of dummies, 4.95 m above ground). A dummy was made of a black aluminum bar (3.5×1.1 cm) with inserted pheromone impregnated glass rods. A video camera was fixed next to the central axis on each arm, facing toward the dummies. Cameras monitored an area of about 1 m² in the plane of a dummy with the dummy in its center.

Pheromone Components and Artificial Blends. 9-ODA, 9-HDA, and 10-HDA were obtained from Phero Tech Inc. (Delta, BC, Canada) and HOB was obtained from Sigma-Aldrich (Taufkirchen, Germany). All compounds were dissolved in absolute ethanol. Test blends were mixed according to published data on the composition of the mandibular secretion in *Apis mellifera* virgin queens (Plettner et al., 1997). The complete artificial virgin queen blend (virgin queen equivalent = Qeq_{virgin}) contained the following: 70.4 µg 9-ODA, 12.6 µg 9-HDA, 76.7 µg 10-HDA, and 0.3 µg HOB. Test blends differed in the number of components. Dose-response tests showed that a dose of 0.1 Qeq_{virgin} was adequate to attract a sufficient number of drones at our experimental site (see Results). Glass bars (10–12 mm in length) with a small groove in which 10 µl pheromone blends dissolved in ethanol were deposited were used as pheromone dispensers. The solvent was allowed to evaporate for 5 min. Then the glass rods were wrapped in aluminum foil and kept on ice until use. Immediately before the field tests started, the glass rods were fixed to the aluminum dummies.

Two-choice Experiments. On each experimental day, five to nine test runs were conducted with different pairs of pheromone blends in random order, depending on drone abundance. Each test lasted for 15 min, of which the first 5 min were used to attract drones to the carousel and were excluded from the data analysis. Prior to each test run, the video cameras were synchronized to analyze identical periods for both dummies. Altogether, we conducted 86 tests (out of which 63 were included in the analysis, see below) with five different pairs of

pheromone blends: 9-ODA vs. 9-ODA ($N = 11$; control experiment); 9-ODA vs. 9-ODA + 9-HDA ($N = 11$); 9-ODA vs. 9-ODA + 10-HDA ($N = 13$); 9-ODA vs. 9-ODA + 9-HDA + 10-HDA ($N = 13$); and 9-ODA vs. 9-ODA + 9-HDA + 10-HDA + HOB ($N = 15$). The number of drones attracted to each dummy during the 10-min test interval was determined by counting the number of drones every 20 sec, i.e., on 31 video freeze images per test. Because the numbers of drones attracted to the carousel differed strongly among experimental runs (see Figure 3A), we calculated the relative number of drones attracted to each of the two dummies for each test by taking the sum of all drones that were counted on the 62 images from both sides per test as 100%. Only test runs that attracted at least 10 drones to the carousel were included in the analysis. The numbers of drone contacts with the dummies were determined by counting all contacts during each 10-min test interval. Only intense and unambiguous contacts with the dummy, i.e., clinging to the dummy in a clear mounting attempt, were counted. Again, to allow comparisons between tests despite the variation in absolute numbers of drones present in the vicinity of the carousel, we calculated the relative number of contacts to each of the two dummies for each test run. Only test runs in which at least five contacts were scored were included in the final analysis. To test for significant differences of drone numbers or numbers of contacts between the two arms, we applied the Wilcoxon signed-rank test for each of the five pairs of pheromone combina-

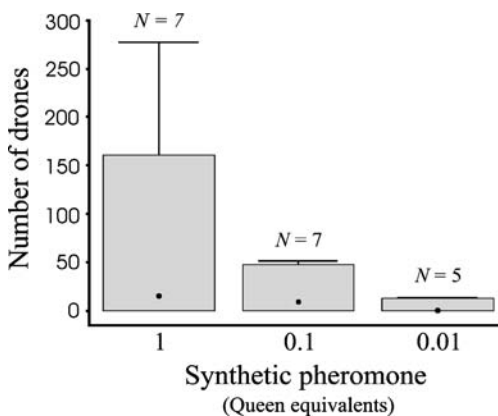


FIG. 2. Effect of different concentrations of synthetic queen pheromone on the attraction of drones to the carousel. One virgin queen equivalent contained 70.4 μg 9-ODA, 12.6 μg 9-HDA, 76.7 μg 10-HDA, and 0.3 μg HOB. Treatments were presented on a dummy at one arm of the carousel. Box and whisker plot indicates median (black dot), first and third quartile (box) and maximum number (whisker) of drones attracted to the dummy per 10-min experimental run.

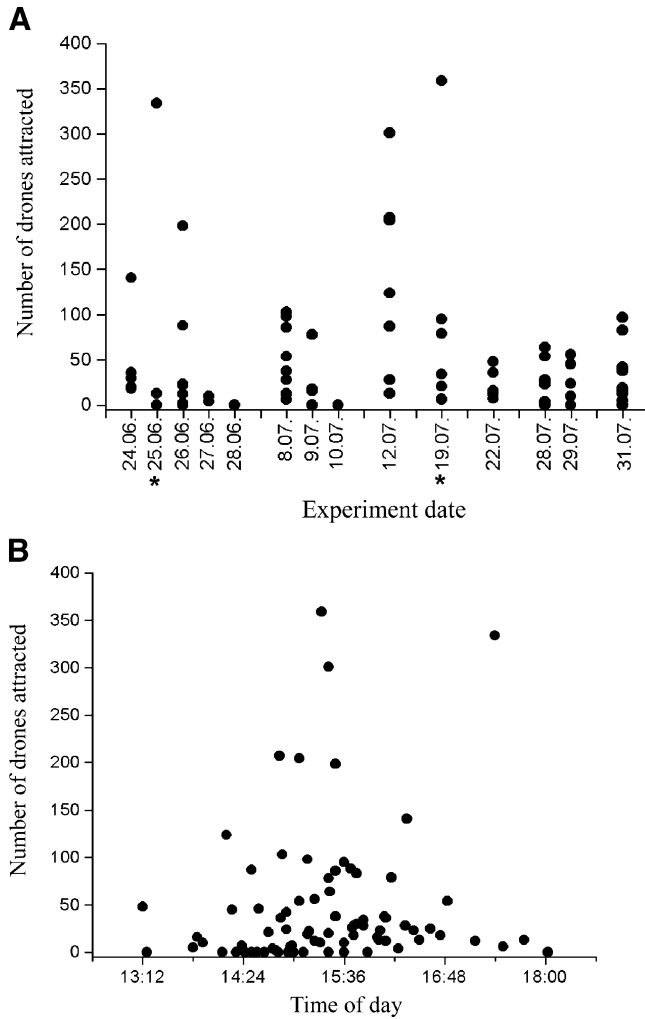


FIG. 3. (A) Number of drones attracted on different days. Each dot indicates number of drones per 10-min experimental run (median = 23, first quartile = 7.3, third quartile = 56, minimum = 0, maximum = 1248). During two experimental runs, a large number of drones were attracted: 528 drones (*) on June 25, and 1248 drones (*) on July 19. These two data points were omitted from both figures. (B) Effect of time of day on the attraction of drones. Dots indicate the number of drones attracted per run for all experimental replicates.

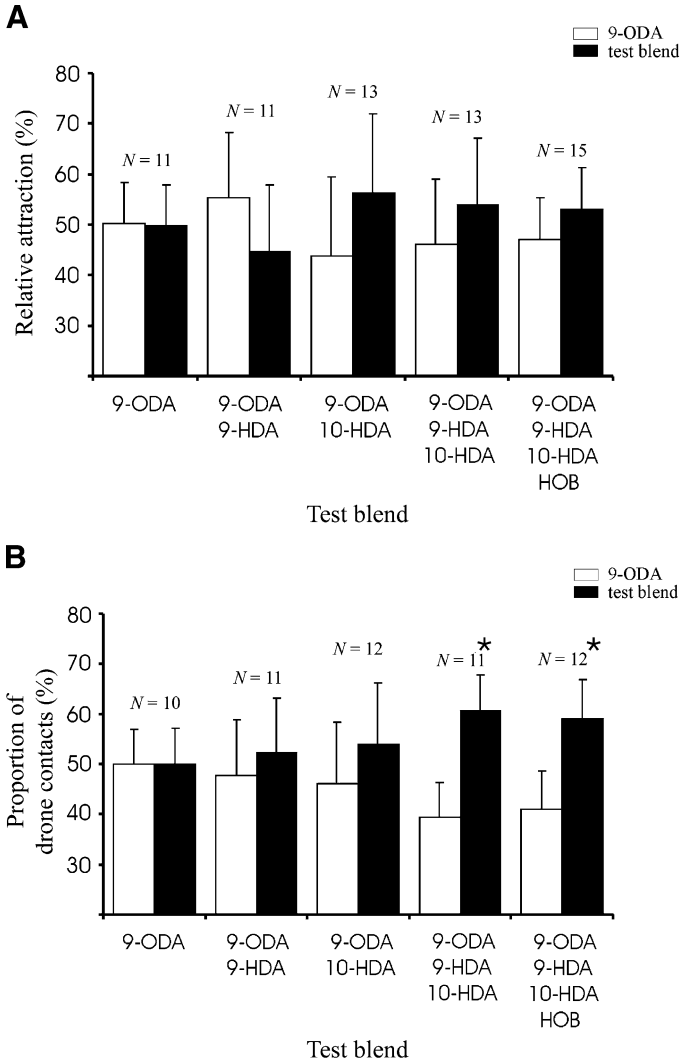


FIG. 4. (A) Proportion of drones attracted (\pm SD) to the vicinity of the treatment and control lures. (B) Proportion of drone contacts with the treated and control dummies. White bars, 9-ODA only; black bars, test blends. *Significant differences between 9-ODA and the blend ($P < 0.05$).

tions. All data were arcsine-transformed prior to analysis. *P* values exceeding 0.05 were considered as not statistically significant.

RESULTS

Attraction of drones to a synthetic pheromone lure was locally restricted to an area of ~100 m in diameter (Figure 1). The distance to the closest honeybee colonies was about 150 to 300 m. The number of drones attracted to the drone carousel was pheromone dose dependent (Figure 2), demonstrating that drones were attracted to treated dummies by olfactory, rather than visual, cues. Numbers of drones attracted to the carousel were stable during each test run but varied among test runs and among test days (Figure 3A,B).

To ensure that there was no bias between the two dummies, we first treated both lures with identical doses of 9-ODA, which resulted in equal numbers of drones being attracted to both dummies (Figure 4A). In subsequent trials, 9-ODA as a single substance was as attractive as any other combination of 9-ODA and minor components (Figure 4A).

We also found no evidence for any asymmetry in drone contacts when equal amounts of 9-ODA were presented on both arms (Figure 4B). Adding 10-HDA or 9-HDA to 9-ODA did not increase the number of contacts compared to 9-ODA alone. However, blends that were more similar to the natural pheromone composition (9-ODA + 9-HDA + 10-HDA or 9-ODA + 9-HDA + 10-HDA + HOB) resulted in significantly higher numbers of contacts with the queen dummy containing the blend compared to the dummy with 9-ODA alone ($Z = -2.81$, $P = 0.005$, and $Z = -2.85$, $P = 0.004$, respectively; Figure 4B).

DISCUSSION

Mating behavior in honeybees is highly competitive between drones, much more than in most other insects. In all honeybee species, drones and virgin queens leave their colonies for mating flights at species-specific times of the day. In our experiments, drones were present at the drone congregation area between 1:00 and 6:00 P.M., with a peak time around 3:30 P.M. Drones gather at the drone congregation areas (estimated sizes range from 50 to 200 m in diameter), waiting for virgin queens to appear (Ruttner, 1985). Upon detection of the queen's sex pheromone, drones initiate their visually driven chasing behavior, all competing for the queen and only a few being successful. To catch a queen, the drones must be fast and precise in detecting and chasing. Although detailed analysis of honeybee mating behavior is still lacking, one may assume that the

chasing behavior of drones is based on a succession of different behavioral steps including detection of the pheromone, searching the sky for small dark flying objects, a rapid pursuit of the queen (Gries and Koeniger, 1996), and finally successful grasping and mounting of the queen.

Our experiment to determine the location of a drone congregation area showed that the effective range of the pheromone was limited to a circular area with a diameter of ~100 m. We assume that the location of this drone congregation area is stable over the years, because of previous reports that one could attract drones with pheromone lures in that area (J. Tautz, pers. comm.). The closest beehives (marked by an “H” in Figure 1) were located about 150–300 m north, with entrances facing south in the direction of the drone congregation area. The most obvious visual feature of the site is the boundary of the orchard and the open meadow, and this may be one of the constitutive visual cues. If drones from the specified colonies join this drone congregation area, the boundary between the orchard and the meadow might function as a visual “stop and stay” stimulus; however, this remains to be investigated.

Our drone carousel experiments demonstrated that in addition to 9-ODA (the so-called queen substance), other components of the queen mandibular gland secretion, particularly 9-HDA and 10-HDA, play a role in pheromone communication between queens and drones. The latter two compounds are not attractive to drones from a distance (Loper et al., 1996), but added to 9-ODA they increased the drone’s contacts with a queen dummy. Whether HOB also mediate drone behavior cannot be inferred from our experiments. The effect of 9-HDA and 10-HDA on the frequency of mounting suggests that 9-HDA and 10-HDA only act over a short range. However, we can not exclude the possibility that these compounds are active at long range (tens to hundreds of meters), because our experimental set-up did not specifically test for those distances. A similar increase in the frequency of mounting behavior in drone carousel experiments was also found when tergite gland extracts were added to 9-ODA (Renner and Vierling, 1977). The conclusion from both experiments is that adding either 9-HDA and 10-HDA or tergite gland secretions to 9-ODA increases the effectiveness of the artificial stimulus, leading to a stronger response and a more complete performance of the mating behavior sequence. Whether the components of the mandibular gland and the tergite gland have distinct functions in the sequence of the mating behavior remains unknown.

It is intriguing to compare our findings on sex pheromone communication with those on queen–worker communication. Slessor et al. (1988) and Keeling et al. (2003) showed that a blend of 9-ODA, 9-HDA, HOB, and HVA similar to that found in mated queens elicited worker retinue behavior. 10-HDA, which is reduced in quantity in mated compared to virgin queen glands, does not play any role in queen–worker communication (Slessor et al., 1988). In contrast to

those experiments, we found clear evidence that 10-HDA, which is produced in large amounts by virgin queens, does have a pheromone function in mating behavior. Taken together, the data suggest that *A. mellifera* queens produce different signals during their adult life stages in relation to different communication contexts. As virgin queens they produce a signal to attract drones on mating flights, and as mated queens they produce a signal for the workers. The signals are produced by the same gland and comprise almost identical substances, but the relative composition changes between virgin and mated queens. This change in mandibular gland composition may be induced by successful mating.

9-ODA is viewed as the major long-distance sex attractant for all honeybee species (Butler et al., 1967; Ruttner and Kaissling, 1968; Shearer et al., 1970; Sanasi et al., 1971; Koeniger and Koeniger, 2000), and it is the main component in the queen mandibular glands of all species except *A. florea* (Plettner et al., 1997), in which 10-HDA is the most abundant mandibular gland component. Our finding that the two other most abundant decenoic acids also have a pheromonal function in *A. mellifera* suggests that they might have sex pheromone functions in other honeybee species as well (Brockmann and Brückner, 2005). Thus, it is likely that 10-HDA is a sex pheromone component for *A. florea*, and it might even be the major functional pheromone component, replacing 9-ODA in this species. In addition, differences in the relative composition of mandibular gland components might be effective in reproductive isolation between sympatric species. GC analyses show that the relative composition of mandibular gland components varies among different *Apis* species (Plettner et al., 1997). Whether drones use these relative differences to discriminate between queens of their own and sympatric species remains to be determined.

Acknowledgments—We thank G. Eisenmann and N. Schneider, who built the drone carousel, and K. Galmbacher and P. Bernbauer, who helped with data collection. J.S. and A.B. were supported by the German Research Foundation DFG (SFB554 and Graduiertenkolleg 200).

REFERENCES

- AYASSE, M., PAXTON, R. J., and TENGÖ, J. 2001. Mating behavior and chemical communication in the order Hymenoptera. *Annu. Rev. Entomol.* 46:31–78.
- BOCH, R., SHEARER, D. A., and YOUNG, J. C. 1975. Honeybee pheromones: Field tests of natural and artificial queen substance. *J. Chem. Ecol.* 1:133–148.
- BROCKMANN, A. and BRÜCKNER, D. 2005. Drone antennae and the evolution of sex-pheromone communication in honeybees. *Indian Bee J.* 65:131–138.
- BROCKMANN, A., BRÜCKNER, D., and CREWE, R. M. 1998. The EAG response spectra of workers and drones to queen honey bee mandibular gland components: The evolution of a social signal. *Naturwissenschaften* 85:283–285.

- BUTLER, C. G. 1971. The mating behavior of the honeybee. *J. Entomol. (A)* 46:1–11.
- BUTLER, C. G., CALAM, D. H., and CALOW, R. K. 1967. Attraction of *Apis mellifera* drones by the odours of the queens of two other species of honeybees. *Nature* 213:423–424.
- CHRISTENSEN, T. A. 1997. Anatomical and physiological diversity in the central processing of sex pheromone information in different moth species, pp. 184–193, in R. T. Cardé and A. K. Minks (eds.). *Insect Pheromone Research. New Directions*. Chapman and Hall, New York.
- FREE, J. B. 1987. *Pheromones of Social Bees*. Comstock, Ithaca, NY.
- GARY, N. E. 1962. Chemical mating attractants in the queen honey bee. *Science* 136:773–774.
- GARY, N. E. and MARSTON, J. 1971. Mating behavior of drone honeybees with queen models (*Apis mellifera* L.). *Anim. Behav.* 19:299–304.
- GRIES, M. and KOENIGER, N. 1996. Straight forward to the queen: Pursuing honeybee drones (*Apis mellifera* L.) adjust their body axis to the direction of the queen. *J. Comp. Physiol. A* 179:539–544.
- KEELING, C. I., SLESSOR, K. N., HIGO, H. A., and WINSTON, M. L. 2003. New components of the honey bee (*Apis mellifera* L.) queen retinue pheromone. *Proc. Natl. Acad. Sci. USA* 100:4486–4491.
- KOENIGER, N. and KOENIGER, G. 2000. Reproductive isolation among species of the genus *Apis*. *Apidologie* 31:313–339.
- LINN, C. E. JR., CAMPBELL, M. G., and ROELOFS, W. L. 1986. Male moth sensitivity to multi-component pheromones. Critical role of female-released blend in determining the functional role of components and active space of the pheromone. *J. Chem. Ecol.* 12:659–668.
- LOPER, G. M., TAYLOR, O. R. JR., FOSTER, L. J., and KOCHANSKY, J. 1996. Relative attractiveness of queen mandibular pheromone components to honey bee (*Apis mellifera* L.) drones. *J. Apic. Res.* 35:122–123.
- PHELAN, P. L. 1992. Evolution of sex pheromones and the role of asymmetric tracking, pp. 265–314, in B. D. Roitberg and M. B. Ismam (eds.). *Insect Chemical Ecology. An Evolutionary Approach*. Chapman and Hall, New York.
- PLETTNER, E., SLESSOR, K. N., WINSTON, M. L., and OLIVER, J. E. 1996. Caste-selective pheromone biosynthesis in honeybees. *Science* 271:1851–1853.
- PLETTNER, E., OTIS, G. W., WIMALARATNE, P. D. C., WINSTON, M. L., SLESSOR, K. N., PANKIW, T., and PUNCHIHEWA, P. W. K. 1997. Species- and caste determined mandibular gland signals in honeybees (*Apis*). *J. Chem. Ecol.* 23:363–375.
- RENNER, M. and VIERLING, G. 1977. Die Rolle des Taschendrüsenpheromons beim Hochzeitsflug der Bienenkönigin. (The secretion of the tergite glands and the attractiveness of the queen honeybee to drones in the mating flight). *Behav. Ecol. Sociobiol.* 2:329–338.
- RUTTNER, F. 1985. Reproductive behaviour in honeybees, pp. 225–236, in B. Hölldobler and M. Lindauer (eds.). *Fortschritte der Zoologie*, Vol. 31. Gustav Fischer Verlag, Stuttgart.
- RUTTNER, F. and KAISLING, K.-E. 1968. Über die interspezifische Wirkung des Sexuallockstoffes von *Apis mellifica* und *Apis cerana*. *Z. Vergl. Physiol.* 59:362–370.
- SANASI, A., RATULU, G. S., and SUNDARA, G. 1971. 9-Oxodec-*trans*-2-enoic acid in the Indian honey bees. *Life Sci.* 10:195–201.
- SHEARER, D. A., BOCH, R., MORSE, R. A., and LAIGO, F. M. 1970. Occurrence of 9-oxodec-*trans*-2-enoic acid in queens of *Apis dorsata*, *Apis cerana*, and *Apis mellifera*. *J. Insect Physiol.* 16:1437–1441.
- SLESSOR, K. N., KAMINSKI, L. A., KING, G. G. S., BORDEN, J. H., and WINSTON, M. L. 1988. Semiochemical basis for retinue response to queen honey bees. *Nature* 332:354–356.
- VELTHUIS, H. H. W. 1985. The honeybee queen and the social organization of her colony. *Fortschr. Zool.* 31:343–357.
- WILSON, E. O. 1971. *The Insect Societies*. Harvard University Press, Cambridge, MA.