ABSTRACT

ELIYAHU, DORIT. Chemical Communication in the German Cockroach: Pheromones and Heterospecific Courtship Eliciting Compounds. (Under the direction of Coby Schal).

Sexual communication is vital for the reproductive success of many species. Chemical communication is considered highly effective in being both species-specific and indicative of reproductive status. However, there are cases in which this intraspecific intersexual system breaks down, either by sexual mimicry or by convergence on similar compounds in different species. In the first chapter I review the literature for cases where courtship displays are chemically elicited by non-reproductive conspecifics and by heterospecifics. I group cases as being (1) adaptive to courting males; (2) maladaptive to courting males; or (3) incidental consequences of convergence on similar courtship releasing cues of no adaptive value to either participant in courtship.

Volatile sex pheromones, responsible for bringing of the sexes together, have been studied extensively. On the other hand, contact sex pheromones which are vital for reproductive success in many species, have received much less attention and little is known about their chemistry and biochemical and hormonal regulation. An exception is the German cockroach, *Blattella germanica*, whose contact sex pheromone has been extensively studied in relation to endocrine regulation of pheromone biosynthesis. Here I report on investigations of chemical structure-activity relationship in the German cockroach using naturally occurring and synthetic courtship eliciting compounds.

I show that contrary to expectation, the naturally occurring stereoisomer of the pheromone, (3S,11S)-dimethylnonacosan-2-one, is the least active of the four possible stereoisomers. Extensive behavioral assays with synthetic pheromone analogs and the natural pheromone were conducted to validate this result. Next I identify two additional contact sex pheromone components, predicted from the proposed biosynthetic pathway, and confirm their behavioral activity with synthetic compounds. I used a behavioral-assay guided approach whereby fractions from flash column chromatography, high
performance liquid chromatography and gas chromatography were behaviorally assayed and the active components identified with mass spectrometry.

In addition, I describe two intriguing phenomena where courtship is directed toward non-receptive individuals: the German cockroach male courts immatures of its own species, as well as members of five other cockroach species. I used a similar behavior-guided chemical fractionation approach to purify and identify from nymphs compounds responsible for eliciting courtship. The results show that last instar female nymphs share common pheromonal components with the adult female contact sex pheromone. I also isolated a different, yet to be identified compound, or set of compounds, from young nymphs and last instar female nymphs that elicit courtship in *Blattella* males. The nymphs may elicit courtship as a potentially adaptive strategy of sexual mimicry, whereby they avoid aggression from males or gain nutritional benefits from the courting male. Five other cockroach species can elicit courtship in German cockroach males, and the courtship eliciting compounds in *Blatta orientalis* were identified as related to, but different from any of the sex pheromone components of *B. germanica*. This suggests that the German cockroach male responds to a broader range of chemical structures than necessary for sexual and species recognition. Possible conditions favoring this broad sensory tuning are discussed.
CHEMICAL COMMUNICATION IN THE GERMAN COCKROACH: 
PHEROMONES AND HETEROSPECIFIC COURTSHIP ELICITING COMPOUNDS

by
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BIOGRAPHY

I was born and raised in a little Israeli type of settlement called a "moshav". It was far away from anywhere civilized, so I ended up spending most of my spare time in the fields and in our back yard, finding injured animals, trying to take care of them, and then burying them when those attempts failed, which was mostly the case. These failures motivated me to become a veterinarian, a dream I decided to follow since the age of 10. After serving 2 years in the Israeli army I had to clear my mind by going somewhere far away and exotic, so I went backpacking in South America, where I learned a little bit of Spanish, and saw the most amazing animals, people and places. That was probably the most fulfilling experience I've ever had. Alas, I had to get back and follow my dream. While working toward a B.S. degree in Animal Sciences as part of my preparation for vet school, I took so many entomology courses that my friends predicted I'd be an insect vet, performing surgeries on decapitated praying mantids, and curing sprayed cockroaches (which I did end up attempting – another failure in my veterinary career…). After a year an a half in vet school, which was interesting, but not as fulfilling as I expected, I finally decided to abandon my veterinary dream and switch to the study of insects. I completed my M.S. degree studying pheromone regulation in moths and, like Tom Eisner, wanted to find natural products from insects in tropical forests. I couldn't find anyone who'd train me to do that in Latin America, so I ended up at NC State – a backup plan that wound up being a wonderful experience, ranked very close to my South American adventure.
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Finally, I wish to thank my best friend and companion, Mark Sciabica, for being so loving and tender, for his valuable input to my research (even though he is a mathematician!) and for bringing me smoothies in the lab on the weekend. That's very sweet.

Being away from home was not difficult with all these wonderful people who made me feel at home.

I would also like to acknowledge co-authors for their specific contributions in the following chapters:

• Chapter 2: Professors Kenji Mori and Hirosato Takikawa synthesized the four stereoisomers of 3,11-dimethylnonacosan-2-one. Dr. Walter S. Leal confirmed the stereochemical identity of the stereoisomers.

• Chapters 3 and 4: Dr. Kenji Mori synthesized the 27-hydroxy- and 27-oxo-dimethylheptacosan-2-one.
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CHAPTER 1

Love is Blind:
Sexual Cue-Scrambling and Chemically-Mediated Courtship of
Inappropriate Mates

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SUMMARY
Courtship plays a major role in reproduction and it is often essential for identifying suitable mates: it facilitates the union of receptive individuals of the opposite sex. Chemical communication is among the most specific and indicative courtship channels, yet there are cases where it is exploited by inappropriate mates through chemical mimicry. Furthermore, the specificity can fall apart through metabolically-related processes that are common in many species, and may result in similar by-products that may function as chemical signals. Here we review cases of chemically-mediated intraspecific courtship of inappropriate mates, and non-adaptive interspecific courtship. We find that courtship induced by inappropriate conspecific mates may have an adaptive value either to the suitor or the elicitor. Interspecific courtship might be the result of a breakdown of a physical reproductive isolation barrier. The outcome of such courtship may be devastating to either or both species, and needs to be taken into account when considering the introduction of a biological control agent.
INTRODUCTION
Courtship is often an essential prelude to reproduction, and in many species it serves multiple functions, primary among them is recognition of suitable mates—sexually receptive conspecifics of the opposite sex. Additional functions of courtship include influencing mate choice, investing in reproduction through gift exchange or sexual cannibalism, and initiating endocrine events and receptivity in the courted individual. These multiple facets of courtship can shape the evolution of mating systems, sexual selection, and reproductive isolation. However, there are reports of courtship displays elicited by non-reproductive conspecifics and by heterospecifics. Courtship of non-receptive or inappropriate individuals could be (1) the result of an adaptive process, for example the male may gain courtship experience; (2) maladaptive to the courting male, for example sexual mimicry that bears an adaptive value to the mimic by gaining access to mates, resources, or deflecting aggression; or (3) it may be an incidental consequence of convergence on similar courtship releasing signals and therefore of adaptive value to neither participant in courtship. In this review we focus on chemically-mediated courtship. We start with a general definition of courtship and enumeration of its functions, and then explore cases of exploitation of inter-sexual communication systems by conspecifics. Inter-sexual visual (coupled with behavioral) mimicry is relatively common in animals (e.g., Muller and Wrangham 2002; review: Andersson 1994), but this review focuses on the much less pervasive intraspecific chemical mimicry. Likewise, interspecific sexual mimicry is relatively common in nature, and has been investigated most extensively in plant-arthropod pollination systems (review: Salzmann et al. 2006) and in arthropod-arthropod aggressive sexual mimicry (e.g., review: Haynes and Yeargan 1999), systems that we exclude from this review. However, we review cases of apparently non-adaptive interspecific sexual interaction mediated by chemicals, where neither member of a courting pair appears to gain from the interaction. We conclude with suggestions for future research directions and areas of study that could contribute to our understanding of these phenomena, and in turn, may increase our understanding of the role of chemical communication in the evolution of mating systems.
We reason that unraveling the phenotypic structure of courtship and mechanisms that underlie sexual behavior will lead to better understanding the evolutionary forces that mold features of the chemical communication channel, such as spectrum breadth and response thresholds.

COURTSHIP AND ITS FUNCTIONS
Courtship is defined as the heterosexual reproductive communication system leading up to the consummatory sexual act (Morris 1970). As such, the main functions of courtship are to attract and arouse members of the correct species, of the opposite sex, in a synchronized manner to the arousal of the suitor (Morris 1970). For example, in the terrestrial salamander *Plethodon jordani* the male courtship pheromone increases the receptivity of the female during courtship (Rollmann et al. 1999). In the amphibian salamander *Taricha granulosa* the enhanced receptivity is achieved by elevation of gonadotropin levels in the brain (Propper and Moore 1991).

In order to attract and arouse appropriate mates, components of courtship behavior are often very species-specific, and in many cases serve as prezygotic reproductive isolation mechanisms (Boake 2002). The wing vibration courtship song in *Drosophila* spp., for one, is often species-specific (O'Dell 2003). Additional specificity is provided by the contact pheromones on the female’s cuticle, as in the case of *D. simulans* males, that require the conspecific sex pheromone for initiation of courtship (Boake 2002).

Moreover, courtship facilitates bringing the sexes to the right place. This is obvious with territorial animals, but holds true in non-territorial species, such as whitefish, that display only in specific localities, and other species of fish whose eggs can only hatch on specific substrates (Bastock 1976). In insects, courtship and mating on host plants can place the female on the proper oviposition substrate.

Courtship also serves as an advertisement for the value of the suitor. Females in general invest more parentally, and therefore sexual selection is often the result of female choice. Display of costly male traits during courtship, that can influence female
choice, may reflect some underlying measure of quality (Halliday 1983). Through mathematical modeling it was found that costly gifts (or costly displays), either in a facultative paternal care model, or one without paternal care—even when these gifts are worthless to the female—and perhaps because of that—act as a credible signal of the male’s quality (Sozou and Seymour 2005).

Examples for nuptial gifts in the form of feeding supplied by males during courtship are abundant, especially in arthropods. The gifts can be captured prey, glandular secretions, parts or even the whole male’s body (Vahed 1998; Vahed 2007). Often the gifts serve as a mating effort: to attract the female, guard/monopolize her, facilitate coupling, and/or to maximize ejaculate transfer. Vahed (1998, 2007) did not find much support for high nutritional value in these nuptial gifts, but robust design that measure fecundity and longevity are sorely lacking. Many cockroach species display courtship by raising their wings and exposing specialized glands on the dorsum of their abdomen that contain nutrient secretions (Roth and Willis 1952; Nojima et al. 1999). In most cockroach species these glands are broadly distributed and their meager secretions likely do not provide any nutritional value to the females. But in some species, the glandular cells have aggregated to form large secretory reservoir that might serve as both a nutritional gift and an indicator of the male's fitness. However, the main function of these secretions is to facilitate mating: A female feeding on the secretions is aligned correctly for copulation (Roth and Willis 1952; Nojima et al. 1999; Kugimiya et al. 2003). In some predatory species, where cannibalism is common, such as spiders and mantids, courtship often serves as an appeasement tool for the male in an attempt to avoid predation (Kynaston et al. 1994).

Courtship may also function to repel conspecifics of the same sex, which is more common in territorial species, and is often very similar to threat displays. In some cases, the male displays this behavior toward males, immature females, which are deterred and sexually mature females. The mature females are not deterred, but rather attracted and stimulated, as is the case of the domestic hens, Gallus spp., and the river bullhead, Cottus gobio. Deterring other males with courtship displays may facilitate courtship, as
some females will not mate in the presence of other suitors (Bastock 1976). In many moth species, the males produce a close-range courtship pheromone, released from their hairpencils that serves as an aphrodisiac to the courted female but also repels potential mating competitors (Hillier and Vickers 2004; Hillier et al. 2007).

Hence, courtship is often crucial for reproductive success, and its benefits supersede the costs—energy investments (especially in cases where food is supplied) and conspicuousness to predators during display (Pruden and Uetz 2004). Among sexual communication channels, chemical communication is considered to be highly effective in being both species-specific and indicative of the reproductive status of the emitter of the sex pheromone (Roelofs 1995; Rafaeli 2002). Nonetheless, there are cases in which organisms exploit this intersexual species-specific communication system for their own benefit. The exploiter can be a non-reproductive or otherwise inappropriate mate of the same species, or a heterospecific (review of the latter: Haynes and Yeargan 1999). Other cases exist where non-adaptive interspecific courtship occurs, with potential detrimental effects for both parties.

COURTSHIP INDUCED BY INAPPROPRIATE CONSPECIFIC MATES

Sexually unreceptive adult females elicit courtship in adult males

When the two sexes are attracted to each other over long distances, specific “calling” behaviors advertise both the location and sexual receptivity of the calling sex. Thus, female moths and cockroaches, for instance, emit volatile pheromones only when they seek to be mated, and this behavior is suppressed by mating (Rafaeli 2002, Schal et al. 2003). Inappropriate courtship of mated females would be rare in these systems, except perhaps under crowded conditions. Where courtship is mediated by cuticular pheromones, there is growing evidence that both receptive and unreceptive females are attractive to males—long-chain lipids are used that cannot be rapidly “turned off”.

The persistence of courtship of sexually mature but unreceptive females—for example, of already mated females—is likely a consequence of the relatively high abundance and multiplicity of functions of chemical signals that mediate contact
communication systems. Medium- and long-chain cuticular hydrocarbons, and related more polar lipids, function primarily to prevent water loss in terrestrial arthropods, and to do so they are highly abundant (up to hundreds of micrograms; Howard and Blomquist 2005). A single compound, or a subset of these lipids, can also serve as sexual signals. Indeed, cuticular pheromones may constitute an excellent example of a sexually dimorphic trait that functions in sex recognition and courtship that might have been secondarily coopted from pre-existing functions (West-Eberhard et al. 1987; Sherratt and Forbes 2001). Rather than turning off this intricate, highly pleiotropic system, the mated female might cope with being courted, as long as the cost to her is low, or engage in behaviors to avoid courting males (e.g., depart from an aggregation).

But do males gain adaptive value by courting already mated females? In most systems, probably not. However, when females normally mate multiply they might be persuaded in courtship to mate again. Another intriguing hypothesis is that males gain sexual experience from such interactions, which then facilitates faster and more effective courtship with virgin females. In Drosophila melanogaster, where courtship is negatively associated with male longevity, experienced males that already courted mated females spent less time doing so the day after than males who had no such experience; hence, the experienced males spent more time courting virgin females than inexperienced males, and this learning process is mediated by cuticular hydrocarbons (Dukas 2005; Ejima et al. 2005). Similar case was found in the threespine stickleback, where males experienced with courting supergravid female dummies, who were punished by simulation of the female's retreat during the training phase, spent less time courting such females than control males during the test phase (Jenkins and Rowland 1997).

**Immature individuals elicit courtship in adult males**

Immature stages include individuals that are pre-pubescent or, in insects, adults that have yet to complete sexual maturation, or juvenile stages that are morphologically and sometime ecologically different from the adults. There are few examples of courtship of
immature stages, but there are several potential adaptive values for such “sexual cue-scrambling” phenomena, including (1) to reduce aggression by adult males; (2) to gain nutritional benefits by mimicking females (e.g., nuptial gifts); and (3) to gain access to other resources, including territories and mates. Nevertheless, there are also cases where no clear adaptive benefit can be attributed to his behavior and it may therefore represent a case of inconsequential use of compounds by immatures that happen to mimic sex pheromones.

The promiscuous nature of courtship behavior of the male German cockroach, *Blattella germanica*, was observed by Roth and Willis (1952). They showed that a large proportion of adult males could be stimulated to display courtship by intact full-grown male and female nymphs as well as by isolated antennae from nymphs and teneral adults that were used to manually stroke the antennae of test males. The courtship display, a clear unmistakable response that occurs only in a sexual context, comprises a turn away from the stimulus coupled with raising the wings and thereby exposing tergal (dorsal) glands with nutritious secretions. More than 20 years before the female contact sex pheromone was identified (Nishida et al. 1974), Roth & Willis (1952) postulated that the courtship inducing substance of nymphs and teneral adults was a product of the molting process and different from the female’s sex pheromone.

Eliyahu et al. (chapter 4) sought to clarify whether courtship was directed only at teneral nymphs or at nymphs of all ages, and to determine whether adult sex pheromones were employed by nymphs. We found that younger as well as last instar nymphs, even many days after they molted, could release courtship in males, but they use different suites of compounds. Last instar female nymphs, which begin the process of sexual differentiation, start producing the contact sex pheromone of adult females, albeit at much lower amounts. This coincides with other adult female-specific morphological and physiological changes that are evident in last instar females, including body size and shape, cell numbers of the corpora allata (CA) which produce the gonadotropic hormone juvenile hormone (JH) (Chiang et al. 1991), production of JH and its release into the hemolymph (Treiblmayr et al. 2006), and differentiation of the
fat body which enables it to synthesize small amounts of vitellogenin (Kunkel 1981). However, early instar nymphs and male nymphs of all stages, which also can elicit courtship, do not possess any of the adult female contact sex pheromone components. Instead, these, teneral adults, and adult females possess an unidentified courtship eliciting compound whose chemical structure is currently under investigation.

Teneral individuals might use courtship stimulation to distract males from attacking them when their cuticle is still soft, their mobility is limited, and they are highly vulnerable in general. Cannibalism of vulnerable insects is very common in natural populations, especially under conditions of food scarcity, and it is thought to serve as a means of demographic regulation (Fox 1975). Sclerotized nymphs, on the other hand, which are not as vulnerable as teneral individuals, may benefit from the nutritional value of the tergal secretions. Courted nymphs readily feed on male glandular secretions (Roth and Willis 1952), and studies in our lab provide preliminary evidence for a significant role of such feeding in survivorship of nutrient-deficient nymphs.

Avoiding aggression appears to be the main function of sexual mimicry displayed in the rove beetle, *Aleochara curtula* (Peschke 1985). In this species adult males have limited access to females, which leads to fierce intrasexual competition and high levels of aggression. An aggressive encounter might result in expulsion from the food source (carrion), or even death. Newly eclosed males, not yet sexually active, avoid such outcome by producing the female sex pheromone, a blend of the hydrocarbons (Z)-7-heneicosene and (Z)-7-tricosene. The response of males to this pheromone is a sexual act of grasping with the parameres. By eliciting sexual responses in mature males, the immature adult males are allowed to continue feeding, which is essential for their maturation, and they appear undisturbed by mating attempts made by mature males. Although the female pheromone on immature adult males releases attacks from mature females, these attacks are much weaker than male aggression and do not carry grave consequences. Males stop producing the female sex pheromone as they sexually mature, and thus are accepted by females (Peschke 1986).
Competition for mates is thought to be the cause for sexual mimicry in the parasitoid wasp *Lariophagus distinguendus* (Steiner et al. 2005). In this species, females mate only once, immediately after emergence, at their emergence site. Female pupae produce a close-range pheromone that attracts males, and the male guards the pupa until the adult female emerges. This results in an operational sex ratio that is skewed against males, providing a clear advantage to early-emerging males. Hence, late-emerging males are thought to have evolved a mechanism to distract early-emerging males from finding females: male pupae produce the courtship pheromone and thus engage the male-seeking males in futile guarding; in the meantime, the female-mimicking males emerge from the pupae and are ready to find appropriate mates. Males that were killed shortly after emergence were still attractive and elicited courtship in males 72 hrs later. In contrast, 72 hrs old live males did not elicit any courtship behavior in other adult males. This provides evidence for the degradation of the pheromone in males, which would be advantageous for acquiring mates and avoiding molestation by other males (Steiner et al. 2005).

The southern masked chafer, *Cyclocephala lurida*, represents an example where no clear adaptive benefit can be attributed to sexual mimicry, which may be a case of inconsequential use of compounds by immatures that happen to mimic sex pheromones. Grubs of this species live underground, feeding on roots, while adults live above ground and do not normally encounter grubs. But grubs infected with milky disease (*Bacillus popilliae*) prematurely surface above ground and can attract males, which are normally attracted to volatile adult female pheromones that have yet to be identified (Haynes et al. 1992). Males that are attracted to grubs of either sex attempt to copulate with them as they would with sexually mature females. Attraction is mediated by volatile chemicals, as established by activity of field traps baited with whole grubs or with solvent extracts of grubs. Haynes et al. (1992) postulate that non-communicative chemicals in the grubs evolved to be used for sexual communication in adults, where males would lose them over time, while females would co-opt the chemicals as sex pheromone. Whether or not this hypothesis is testable, chemical identification of the female sex pheromone and the
grub pheromone mimic will be a necessary first step. Preliminary gas-chromatographic-electroantennographic results suggest that at least one active component is shared by grubs and females. The grub extract also attracted a congener, *C. borealis*, but extracts of other scarabaeid beetle larvae (Japanese beetles and green June beetles) did not show any activity on *C. lurida* males, indicating that there is some phylogenetic specificity to the pheromone and that comparative studies might be fruitful (Haynes and Potter 1995).

![Figure 1.1 Attractive grubs: (a) males of the southern masked chafer, *C. lurida*, in an attempt to mate with a *C. lurida* larva; (b) male of the northern masked chafer, *C. borealis* in an attempt to mate with a *C. lurida* larva. Photos kindly provided by Kenneth Haynes.](image)

**Sexual mimicry by sexually mature individuals**

Males of many species direct substantial courtship effort to other males (Bagemihl 1999), but the cues that elicit intrasexual courtship, and their relationship to female-produced signals, are generally poorly understood. When practiced by all members of a species, intersexual mimicry results in sexual monomorphism, a controversial mating system best represented by the spotted hyena (Muller and Wrangham 2002). When practiced by a fraction of the population, however, intersexual mimicry represents an alternative strategy in the scramble competition for mates and is much more common.
and tractable, for example in Anolis female-like male lizards (Trivers 1976), cuttlefish (Hanlon et al. 2005) and garter-snakes (Shine et al. 2003). Only in the latter, however, is chemical signaling manipulated by the mimic.

In the red-sided garter snake, Thamnophis sirtalis parietalis, there is a type of male that elicits courtship behavior in other males. These males, termed “she-males” attain the “taste” of females by producing a sex pheromone that is indistinguishable from the female sex pheromone (Mason and Crews 1985). In addition, she-males acquire the “dress” of females by being more covered in mud than other males, in a similar manner to females. Their body temperature is closer to that of females, which may also render them more attractive (Shine and Mason 2001). However, they do not differ from other males in size. The female contact sex pheromone is a mixture of 13 long-chain (C29–C37) saturated and monounsaturated methyl ketones, and it is expressed on the dorsal surface of adult female snakes during the mating season (Mason et al. 1990). The compounds on she-males responsible for releasing courtship are identical to those of females and they arise de novo, not from contact with females (Mason and Crews 1985).

She-males have more testosterone than either females or males, and when put in the same arena with males and a female, tend to have more mating success than normal males (Mason and Crews 1985). Moreover, in mating balls in the field, they have been observed to distract and confuse other males, thereby getting better access to the female (Mason and Crews 1985). Nevertheless, this strategy remains controversial because under experimental conditions courting males can distinguish females from female-mimicking males in the mating ball, are not confused by similarities between them, and do not misdirect courtship to she-males (Shine et al. 2003). Further observations that she-males attract vigorous courtship only when females are not present, and that she-males cease courting females when he-males commenced vigorous courtship also cast doubt on the adaptive benefit to female mimicry in this system. It remains to be determined whether she-males gain non-sexual advantages (e.g., thermoregulation) by mimicking females.
NON-ADAPTIVE INTERSPECIFIC SEXUAL INTERACTIONS

Sex pheromones are known to be highly specific: some species produce unique molecules, others achieve high level of specificity by using simple molecules in different ratios in a blend (Roelofs 1995; Rafaeli 2002). However, cases of convergence on the same compounds are not uncommon. An extreme example is provided by (Z)-7-dodecen-1-yl acetate, shared by dozens of moth species, as well as by the Asian elephant (Rasmussen et al. 1996). This should not come as a surprise, since all multicellular organisms share common biochemical pathways that may result in similar or identical by-products (Wyatt 2005). Despite the common pheromonal structure, elephants and moths are unlikely to hybridize. Even more closely related species that share the same compound as sex pheromone can avoid interspecific courtship and interbreeding by using different concentrations of the pheromone (Kaae et al. 1973). Here we discuss cases where interspecific mating, with its detrimental effects, may occur due to courtship response to heterospecific sex pheromone.

The German cockroach, *B. germanica*, discussed above with relation to intraspecific interactions, was observed to court antennae taken from several cockroach species, including the oriental cockroach, *Blatta orientalis*, some Orthopterans, and even a Dermapteran and a Lepidopteran elicited courtship in the male (Nishida and Fukami 1983). The male, however, did not court antennae taken from the American cockroach, *Periplaneta americana*, suggesting that despite this apparent promiscuity, the male German cockroach courtship display is quite selective as well. We confirmed this with a survey of male responses to antennae taken from males, females and nymphs of 20 species of cockroaches (Eliyahu et al., Chapter 5). We found that antennae of males, females and nymphs of four species (*Supella longipalpa*, Blattellidae; *B. orientalis* and *P. australasiae*, Blattidae; *Blaptica dubia*, Blaberidae) elicited courtship, while antennae from only females and nymphs of *Gromphadorhina portentosa* (Blaberidae) elicited courtship; male antennae did not. This is probably due to different physical properties of the male antennae. We isolated the active compounds from *B. orientalis* and found them
to be different from the female contact sex pheromone, although they share several common features.

The contact sex pheromone of the female German cockroach is a blend of six hydrocarbon oxidation products (Eliyahu et al., Chapter 3). Hydrocarbons are common on the cuticular surface of insects serving multiple functions, including prevention of desiccation, a barrier for microorganisms, and as intra- and interspecific chemical communication signals (Howard and Blomquist 2005). It is therefore reasonable to assume that similar pathways in various species might operate on these hydrocarbons to create related products. Since all of these courtship-eliciting species do not share the same habitats with the German cockroach, interspecific courtship is unlikely to be an evolutionary force in this system. For the same reason, interspecific courtship behavior does not appear to have an adaptive value, and cannot be considered mimicry.

When geographic reproductive isolation between two closely related species breaks down due to recent introduction of one to the habitat of the other, the result could be devastating for the native population. This may be the case with the brown trout *Salmo trutta*, an introduced species in the habitat of the brook trout *Salvelinus fontinalis* (Grant et al. 2002). Females of both species exhibit normal spawning behavior when attended by males of either species. Moreover, in most cases where the two species co-occur, the majority of females are attended by males of both species. In both of these species the olfactory system was found to be sensitive to the same putative pheromone, prostaglandin F$_{2a}$ and its derivatives (Essington and Sorensen 1996), which might contribute to the cross-spawning observed.

This level of interspecific sexual interaction may result in reduced reproductive success through wasted gametes and by diverting fish from interacting with conspecifics. Furthermore, the larger size of the brown trout might be advantageous during mate competition, and this might lead to the elimination of the native brook trout (Grant et al. 2002).

In a similar way, introduction of an exotic biological control agent into the habitat of a closely-related native species could have detrimental effect on the native
population, and reduced biocontrol efficiency, if interspecific courtship occurs. *Eretmocerus mundus* is a native to the Mediterranean basin and is considered the most important whitefly controlling agent in greenhouses in southern Spain. *E. eremicus* is native to the United States and is an effective biocontrol agent there. Both species produce long-range and non-volatile, short-range pheromones which were found to be cross-attractive; male *E. eremicus* were attracted to the *E. mundus* female pheromone (Ardeh et al. 2004). Although the males courted heterospecific females, females did not accept them, due to differences in courtship behavior. However it is of importance to be aware of the possibility of hybridization when designing biological control programs.

**FUTURE DIRECTIONS**

Reports on courtship of unsuitable mates may not be abundant, but that does not necessarily indicate scarcity of occurrence in nature. Such events may not always be obvious, especially when not looked for. Colony maintenance in the laboratory may also reduce opportunities to observe such phenomena; often reproductive adults are kept separately from immatures. Therefore, if we are to learn more about the extent to which courtship toward unsuitable mates occurs in nature, we have to look more closely at reproductive behavior in the field. Furthermore, it is important to consider this when studying the effect of introduced species on closely related native species.

The cases presented in this review also need to be further investigated. In many of them the identity of the courtship inducing pheromone mimics is yet to be elucidated and compared to that of the sex pheromone. Such findings can shed light on structure-activity relationships in the reception of pheromone by the courting males.

Moreover, identification of heterospecific courtship cues may help in understanding biochemical pathways and their function. For example, it does not appear that the compounds from *B. orientalis* which elicit courtship in the German cockroach serve as sex pheromones in the former, as they are found both in males and females, as well as in nymphs (chapter 5). The German cockroach female-specific biosynthetic
pathway of converting the hydrocarbon to a ketone might therefore be non sex-specific in other cockroach species.

Thus, elucidating the phenotypic structure of courtship and the mechanisms that underlie sexual behavior may lead to better understanding of the evolutionary forces that mold the features of chemical communication.

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CHAPTER 2

BEHAVIORAL ACTIVITY OF STEREOISOMERS AND A NEW COMPONENT OF
THE CONTACT SEX PHEROMONE OF FEMALE GERMAN COCKROACH,

Blattella germanica

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Abstract—(3S,11S)-3,11-Dimethylnonacosan-2-one is a major component of the courtship stimulating, contact sex pheromone of the female German cockroach. Although the four synthetic stereoisomers of this compound have been tested in behavioral assays, their relative activity remains unresolved. Using isolated male antennae dosed with synthetic test compounds to assay male behavior, we found that at high doses all four stereoisomers elicited responses from 100% of the males. However, at physiologically relevant doses similar to those found on the female antenna, the (3S,11S)-isomer was the least effective of the four stereoisomers at eliciting courtship responses in males. This is the first example of a natural stereoisomer having less bioactivity than related stereoisomers that do not occur naturally. Another component of the sex pheromone blend, 3,11-dimethylheptacosan-2-one, was previously purified from the female’s epicuticle and behaviorally assayed, but its activity was not confirmed through synthesis. We now confirm that synthetic (3S,11S)-3,11dimethylheptacosan-2-one elicits behavioral responses, but less so than its C29 homolog.
INTRODUCTION

Upon contact with a mature female, sexually mature males of the German cockroach, Blattella germanica (L.), exhibit characteristic courtship behavior that includes an unmistakable raising of the wings and turning away from the female (Roth and Willis, 1952; Nishida et al., 1974; review: Gemeno and Schal, 2004). This act exposes specialized tergal glands on the 7th and 8th abdominal segments of the male, which in turn attract the female to mount the male’s abdomen. As the female feeds on nutrients within reservoirs of the tergal glands, she is properly aligned in a precopulatory position (Nojima et al., 1999). The elicitor of this behavioral sequence is a contact sex pheromone blend composed of several oxygenated derivatives of methyl-branched cuticular hydrocarbons. The most abundant component is 3,11-dimethylnonacosan-2-one (3,11-diMeC29-2one), while two other components, 29-hydroxy-3,11-dimethylnonacosan-2-one and 29-oxo-3,11-dimethylnonacosan-2-one (review: Nishida and Fukami, 1983) are presumably derived from it. The stereochemistry of the natural pheromone was suggested as 3S, by comparison of its optical rotation to that of a methyl ketone with an authentic S-configuration, together with its NMR measurement in the presence of a chiral shift reagent (Nishida et al., 1974). Its 11S-stereochemistry could be rigorously determined only after the synthesis of all four stereoisomers of 3,11-dimethylnonacosan-2-one, followed by comparison of their infrared spectra (as crystals in KBr disks, not as solutions), specific rotations, and melting points to those of the natural pheromone. 500 MHz $^1$H or 125 MHz $^{13}$C-NMR comparisons were useless, all the isomers having shown identical spectra. The final proof of the (3S,11S)-stereochemistry of the natural pheromone rested on the observed lack of melting point depression (mp 44–45°C) in a mixed melting point determination of the (3S,11S)-isomer (mp 44–44.5°C) with the natural pheromone (mp 45–46°C), whereas the mixtures of each of the remaining three stereoisomers with the natural pheromone melted at the range between 33.5–37.5°C (Mori et al., 1981).

Synthesis of the four stereoisomers of 3,11-diMeC29-2-one (Mori et al., 1981)
enabled behavioral assays of their relative activity. Unlike the high degree of stereospecificity demonstrated in the antennae of many insects, in which unnatural stereoisomers are usually less active, or even have an antagonistic effect, the four stereoisomers of 3,11-diMeC29-2-one were shown to be equally active (review: Nishida and Fukami, 1983). However, Abed et al. (1993) reported preliminary observations (data were not presented) that at low doses the (3\text{S},11\text{S})-isomer was more active than the others. The latter assays used higher purity stereoisomers synthesized by Mori and Takikawa (1990). In the present work we conducted dose-response behavioral assays with the four stereoisomers of 3,11-diMeC29-2-one of Mori and Takikawa (1990), in an effort to resolve this discrepancy.

A putative fourth pheromone component, 3,11-dimethylheptacosan-2-one (3,11-diMeC27-2-one), was purified by gas chromatography (GC) from female cuticular lipids and shown to be behaviorally active (Jurenka et al., 1989; Schal et al., 1990). Its gross structure was confirmed by synthesis and mass spectral comparison (Takikawa et al., 1997). We now report on the biological activity of synthetic (3\text{S},11\text{S})-3,11-diMeC27-2-one (Takikawa et al., 1997) and confirm its activity as a component of the sex pheromone.

METHODS AND MATERIALS

Insects. Blattella germanica cockroaches were kept in groups at 27°C under 12:12 light–dark photoperiod and fed dry Purina rat chow and water. Newly emerged adult males and females were separated daily from collectively reared nymphs. Wild cockroaches were collected with a modified vacuum cleaner from an infested commercial pig farm in Warsaw, NC. Adult males were isolated from the collection at least 3 d prior to using them in behavioral assays.
Chemicals and Bioassays. The four stereoisomers of 3,11-diMeC29-2-one were synthesized by Mori and Takikawa (1990) and had >99% diastereomeric excess and ≈100% enantiomeric excess. (3S,11S)-3,11-Dimethylheptacosan-2-one was synthesized by Takikawa et al. (1997). Each compound was dissolved in hexane, and the concentration of each stock solution was confirmed by GC (HP5890II, HP-5 column 30 m × 0.25 mm × 0.25 μm, splitless injection) relative to n-hexacosane as internal standard.

Male behavioral responses were tested using a modification of the “antenna on a stick” assay developed by Roth and Willis (1952). An antenna of a 14–21-d-old adult male B. germanica was excised, attached to a glass Pasteur pipette, and either extracted briefly in hexane to remove male cuticular lipids prior to application of the test compound, or used fresh. A 3-μl hexane solution of a test compound was then applied to the distal 1 cm of the test antenna. The hexane was allowed to evaporate, and the antenna was used immediately to test the responses of 30 males or several groups of 10 males 14–21-d old that were housed individually in 8-cm-ID × 8-cm-deep plastic cages supplied with rat chow and water. All assays were conducted during mid-scotophase, avoiding the first and last 2 hr of the scotophase. Each individual male was tested sequentially. The antennae of each male were gently stroked with the test antenna for up to 1 min, and a positive response was recorded when the male executed a courtship response, rotating his body relative to the stimulus and raising his wings within 1 min. This is an unmistakable response that occurs only in a sexual context and is never elicited by male test antennae unfortified with female pheromone or treated with hexane alone.

Amount of Pheromone on Female Antennae. Antennae from ten 5-d-old females were extracted in hexane containing 100 ng of internal standard (heptacosan14-one). The base of each antenna was prevented from contact with hexane so no internal lipids were extracted. Five groups of 10 paired antennae were extracted. The extracts were reduced under N₂ to 1 μl and injected into a capillary GC column, as above. The GC
oven temperature was kept at 70°C for 1 min, then elevated 30°C per min to 150°C, and 10°C per min to 300°C. The amount of pheromone per antenna was calculated by comparison of the area of the pheromone peak to that of the internal standard.

**Statistical Analysis.** Dose response assays were analyzed using chi-square analysis to find a discriminating dose and pairwise chi-square comparison with Fisher’s exact test within the discriminating dose. ANOVA was used to find differences in responses to various compounds at a single dose. All statistical analyses were performed with SAS (SAS Institute, 2000).

**RESULTS AND DISCUSSION**

*Amount of Pheromone on Female Antennae.* Males generally orient to the female’s antennae before performing courtship. GC-FID analyses indicated that each antenna of 5-d-old adult females contained 0.99 ± 0.12 ng (SEM, N = 5) of 3,11-diMeC29-2-one, the major component of the contact sex pheromone, and 0.409 ± 0.004 ng of 3,11-diMeC27-2-one. It is important to note, however, that female antennae also contain minute amounts of other, more active pheromone components, including the 29-oxo-and 29-hydroxy-analogs of 3,11-diMeC29-2-one. Moreover, by touching other parts of the female body with his antennae, a male would be exposed to as much as 250 ng and 100 ng of 3,11-diMeC29-2-one and 3,11-diMeC27-2-one, respectively (Schal et al., 1990).

*(3S,11S)-3,11-Dimethylheptacosan-2-one.* The synthetic (3S,11S)-3,11-diMeC27-2-one elicited little response from males at 1 ng, but 100% of tested males exhibited courtship responses at doses ≥10 ng (Figure 2.1A). This result confirms that 3,11-diMeC27-2-one is indeed a component of the sex pheromone of *B. germanica* and corroborates previous results showing that natural 3,11-diMeC27-2-one was less active than the C29 methyl ketone (Schal et al., 1990). However, these results indicate that the amount of 3,11-diMeC27-2-one on the female antennae is insufficient by itself to elicit the full sexual response in males. Rather, they indicate that for contact with the antennae
alone to elicit the full courtship response, 3,11-diMeC27-2-one would need to operate in concert with the C29 methyl ketone pheromone, and possibly other components.

3,11-DiMeC29-2-one is derived from the major cuticular hydrocarbon 3,11-dimethylnonacosane (Chase et al., 1990; Schal et al., 2003). The other pheromone components, 29-oxo-and 29-hydroxy-C29, are probably derived, in turn, from 3,11-diMeC29-2-one. Because 3,11-dimethylheptacosane is also a component of the cuticular lipids, it is probable that 3,11-diMeC27-2-one is derived from it. If so, it is possible that the C27 methyl ketone also gives rise to oxidation derivatives that might be present in the pheromone mixture. Because 29-hydroxy-3,11-diMeC29-2-one is ~10-fold more active than the methyl ketone (Nishida et al., 1976), and 29-oxo-3,11-diMeC29-2-one has intermediate activity between these two components (Nishida and Fukami, 1983), it is reasonable to expect that the C27 derivatives, if found, will show structure-activity patterns similar to the C29 pheromone components.

The stereochemistry of the naturally occurring 3,11-diMeC27-2-one has not been established, and because no stereoselectivity was found in the male German cockroach for the major pheromone component, 3,11-diMeC29-2-one (Figure 2.1A), bioactivity of the S,S-isomer does not necessarily demonstrate that it is the natural isomer. Nevertheless, it is reasonable to infer based on the (3S,11S) configuration of the other pheromone components that the 3S,11S configuration is most probable for 3,11-diMeC27-2-one as well (Takikawa et al., 1997).

*Stereoisomers of 3,11-Dimethylnonacosan-2-one.* All four stereoisomers of 3,11-diMeC29-2-one exhibited sharp dose-response curves ranging from background responses to 0.1 ng that were no different from responses to the solvent control, to 100% male response to 3 ng (Figure 2.1A). Surprisingly, however, these dose-response bioassays with carefully calibrated solutions showed that the S,S-isomer of 3,11-diMeC29-2-one was significantly less active than the other three isomers (Figure 2.1A). This was unexpected because the S,S-isomer represents the natural configuration of 3,11-diMeC29-2-one. Nevertheless, this observation was further confirmed with independent assays using freshly calibrated standard solutions and a discriminating dose
of 1 ng loaded on the test antenna; again, 3S,11S-diMeC29-2-one was significantly less active than the other three isomers (Figure 2.1B). This discriminating dose, interestingly, is similar to what is naturally found on a female antenna; and yet, a female antenna rarely fails to elicit courtship behavior in mature males. Therefore, either other pheromonal components are crucial for eliciting this high response, or the female antenna possesses textural/mechanical features that elicit higher sexual responses.

FIG. 2.1. Dose-behavioral response assays of the sexual responses of *Blattella germanica* adult males to four stereoisomers of 3,11-dimethylnonacosan-2-one and to (3S,11S)-3,11-dimethylheptacosan-2-one. In A, 30 males from a lab culture were assayed at each dose, whereas in B, a discriminating dose of 1 ng was used to assay three groups of 30 males each. In C, six groups of 10 males each, collected from a field population, were assayed with two of the stereoisomers. In A, different letters indicate significant differences based on χ² 2 × 2 test of independence with Fisher’s exact test; at all other doses there were no significant differences among treatments. Asterisk (*) indicates significantly higher responses to (3S,11S)-3,11-dimethylnonacosan-2-one than to (3S,11S)-3,11-dimethylheptacosan-2-one, at 3 ng. In B and C, different letters indicate significant differences based on ANOVA; SEM is shown for each mean.
A third independent confirmation was obtained when activity of two of the synthetic isomers, \( R,R \)-and \( S,S \)-, was compared to the natural \( 3S,11S \)-diMeC292-one that was extracted and purified from females by Nishida et al. (1974). The results are shown in Table 2.1. Male responses to the natural and synthetic \( 3S,11S \)-diMeC29-one were similar, but significantly lower (ANOVA, \( P<0.05 \)) than to synthetic \( 3R,11R \)-diMeC29-2-one.

It is possible that these unexpected results were an artifact of working with a cockroach colony that has been maintained in the laboratory for several decades. Therefore, we tested the \( S,S \)-and \( R,R \)-isomers of \( 3,11 \)-diMeC29-2-one on males that were freshly collected from an infestation in the field (Figure 2.1C). The results of this fourth assay were consistent with our previous findings that the natural stereoisomer was significantly less bioactive than one of the unnatural isomers, \( R,R \).

In contrast, previous assays showed no significant differences in the doses of the four stereoisomers that were required to elicit courtship responses in males (Nishida et al., 1979; Nishida and Fukami, 1983). These differences might be due to methodological differences. For example, the enantiomeric purity of the starting material [(\( R \))-citronellic acid] used to synthesize the four stereoisomers in the early studies was \( \approx 92\% \) e.e. Our bioassays used stereoisomers from a more recent synthesis that used enantiomerically pure (\( R \))-citronellol, which resulted in exceptionally pure stereoisomers, especially after careful recrystallization of both the intermediates and the final products (Mori and Takikawa, 1990). Also, Nishida and colleagues used antennae of the cockroach Supella longipalpa as a substrate for testing pheromone isomers and analogs, whereas we used antennae from conspecific B. germanica males. We observed that S. longipalpa antennae have some, albeit low, endogenous courtship-stimulating activity on B. germanica males (D. Eliyahu, preliminary data).
TABLE 2.1. Male sexual responses to male antennae loaded with natural or synthetic female sex pheromone

<table>
<thead>
<tr>
<th>Compound tested (2 ng)</th>
<th>Males responding (%)^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural (3S,11S)-3,11-dimethylnonacosan-2-one</td>
<td>20.7 ± 4.7 a</td>
</tr>
<tr>
<td>Synthetic (3S,11S)-3,11-dimethylnonacosan-2-one</td>
<td>22.0 ± 3.3 a</td>
</tr>
<tr>
<td>Synthetic (3R,11R)-3,11-dimethylnonacosan-2-one</td>
<td>45.0 ± 7.2 b</td>
</tr>
</tbody>
</table>

^a Fourteen groups of 10 males each were tested. SEM is shown for each mean. Different letters indicate significant differences (P<0.05) based on ANOVA.

It is also possible that *B. germanica* male antennae have a compound(s) that masks or inhibits the response to the female pheromone. For example, Nishida and Fukami (1983) found that certain fatty acids inhibit the sexual responses of males to the contact pheromone. In *Nauphoeta cinerea*, the lobster cockroach, a male-specific pheromone (nauphoetin, octadecyl (Z)-9-tetracosenoate) elicits aggressive antennal fencing among males, but also serves as a courtship depressant (Fukui and Takahashi, 1983). Our results might, therefore, be explained if such a compound occurs in *B. germanica*, and if it more specifically inhibits the courtship response to the natural isomer than to the other isomers. To test this hypothesis, dose-response experiments were conducted with male test antennae that were extracted briefly in hexane prior to application of the test compound. These antennae were compared to fresh antennae that were not extracted before application (Figure 2.2). Although pre-extracting the antennae tended to reduce male responses, these depressions were not statistically significant at either low (0.1 and 1 ng) or high doses (10 and 100 ng). This general tendency was likely due to the stiffer and more brittle nature of the test antenna after hexane extraction. However, at a dose of 3.3 ng, extracted antennae that were loaded with the *S,R*-and *R,S*-pheromone stereoisomers stimulated courtship in significantly fewer males than fresh antennae loaded with the same stereoisomers, respectively. Although the same pattern was evident with the *R,R*-and *S,S*-stereoisomers, these minor differences were not
statistically significant. At any rate, the dose-response studies showed, in a fifth independent test, that a synthetic stereoisomer of the natural pheromone (3S,11S) was the least bioactive of the four synthetic stereoisomers (Figure 2.2).

Interestingly, Abed et al. (1993) used the same high-purity stereoisomers that were synthesized by Mori and Takikawa (1990), but they suggested that 3S,11S-diMeC29-2-one was more active than other stereoisomers. Resolution of this discrepancy will have to await publication of the methods and data in support of the brief mention of these preliminary results by Abed et al. (1993).

The importance of stereochemistry in olfactory communication is well known. Pheromones may occur as stereoisomeric mixtures, in which case each isomer may be independently active or the mixture may be most active (Mori, 1998). In most cases, however, production of the pheromone is stereospecific, and so is its reception in the opposite sex, with the natural pheromone isomer being the most bioactive. Indeed, in closely related, sympatrically occurring species, the nonnatural isomer may have antagonistic effects on sexual orientation, as a mechanism of precopulatory reproductive isolation (Millar, 2000). Nevertheless, insects exhibit a wide range of relations between pheromone stereochemistry and pheromone activity, as reviewed by Mori (2002, 2004).

However, to our knowledge, this is the first example where unnatural stereoisomers are more active than the natural stereoisomer. Nevertheless, this observation will require further substantiation with studies of the interaction of each stereoisomer with other pheromone components. For example, activity of the natural stereoisomer may be enhanced by other female pheromone components, whereas activity of the unnatural stereoisomers may not.

To resolve methodological uncertainties, it would be most informative to reexamine the behavior of the previously investigated cockroaches in Japan with the high-purity stereoisomers, to determine whether different populations of the German cockroach produce and respond to different stereoisomers of the contact sex pheromone.
FIG. 2.2. Dose behavioral response assays of the sexual responses of *Blattella germanica* adult males to four stereoisomers of 3,11-dimethylnonacosan-2-one, applied onto extracted, or fresh nonextracted male antennae. For the doses of 1 and 3.3 ng, six groups of 10 males each were tested with each antennal treatment for responses to each of the four stereoisomers; three groups of 10 males each were tested with the other doses. SEM is shown for each mean. Different letters indicate significant differences based on ANOVA.

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CHAPTER 3

Identification of New Contact Sex Pheromone Components of the
German Cockroach, *Blattella germanica*, Predicted from the Proposed
Biosynthetic Pathway

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This chapter consists of a manuscript that will be submitted for publication in *Journal of Chemical Ecology*
Abstract  Upon contacting the cuticular surface of a sexually mature female, the male German cockroach exhibits a characteristic courtship behavior: He turns away from the female and raises his wings, thereby exposing tergal glands. The nutritious glandular secretion stimulates the female to mount the male and feed, thus positioning her appropriately for copulation. A multi-component female contact sex pheromone is responsible for eliciting courtship behavior. The most abundant pheromone components are 3,11-dimethylnonacosan-2-one and 3,11-dimethylheptacosan-2-one, both oxidation products of the abundant hydrocarbon analogs 3,11-dimethylnonacosane and 3,11-dimethylheptacosane, respectively. The C$_{29}$-dimethyl ketone is thought to be further metabolized to give two less abundant pheromone components, 29-hydroxy-3,11-dimethylnonacosan-2-one and 29-oxo-3,11-dimethylnonacosan-2-one. Based on this proposed biosynthetic pathway of pheromone production, we hypothesized that 3,11-dimethylheptacosan-2-one also would be oxidized to give two components, 27-hydroxy-3,11-dimethylheptacosan-2-one, and 27-oxo-3,11-dimethylheptacosan-2-one. Using behaviorally-guided fractionation and chemical analyses of organic extracts of virgin females, and synthesis of the (3S,11S)-isomer of each of the two new components, we now show that the contact sex pheromone of the German cockroach does indeed contain these predicted compounds and thus consists of six components.
Introduction

Sex pheromones of insects are most often produced and emitted as bouquets of related chemicals rather than single-components. Furthermore, in recent years, it has become apparent that most pheromone molecules are produced from the actions of tissue-specific enzymes on intermediates of normal fatty acid metabolic pathways, giving rise, among other compounds, to related hydrocarbons, alcohols, ketones, epoxides, acetates, and aldehydes (reviewed by Howard and Blomquist, 2005). In most cases each of the pheromone components alone is relatively ineffective, but a blend of two or more components may act behaviorally as a “minimal blend”, stimulating attraction and/or courtship behavior (McNeil, 1991). Unlike the vast majority of volatile sex pheromones, however, each of the four known contact sex pheromone components of the German cockroach can independently elicit courtship, and some “minor”, less abundant components are far more active than the major, most abundant components (reviewed by Gemeno and Schal, 2004). Chemical communication in Blattella germanica is, therefore, of interest not only because this species is a pest of major medical and veterinary importance, but also because the chemistry, biochemistry, and behavioral ecology of its sexual communication system is relatively well understood.

Sexually receptive females attract males from some distance with a volatile pheromone, blattellaquinone (Nojima et al., 2005). Upon contact with the antennae and mouth parts, the male perceives the female’s contact sex pheromone on her cuticular surface, and performs a characteristic courtship display. This behavior includes a rotation of the male’s body, turning away from the female so as to orient his abdominal tip toward the female’s head. Thus positioned, he raises the wings to almost 90°, exposing specialized glands on the 7th and 8th tergites. These glands secrete a mixture of lipids, proteins and sugars, that synergistically serve as feeding stimulants (Kugimiya et al., 2002; Nojima et al., 1999). The female then mounts the male to feed on the tergal secretion, and this places her in the correct alignment for copulation (Roth and Willis, 1952). Hence, the contact sex pheromone encodes sex- and species-specific information.
and it triggers and mediates close-range sexual behavior; it is therefore imperative for mating.

The most abundant component of the female contact sex pheromone is (3S,11S)-

\[ \text{(3S,11S)-dimethylnonacosan-2-one (1, Fig. 3.1; Nishida et al., 1974), which is derived through} \]

hydroxylation and subsequent oxidation of the abundant cuticular hydrocarbon 3,11-

\[ \text{dimethylnonacosane (Chase et al., 1992). Based on the well-established biochemical} \]

scheme of conversion of hydrocarbons to methyl ketone pheromones in the housefly

(reviewed by Blomquist, 2003), and presence of the hydrocarbon 3,11-

dimethylheptacosane on the cuticular surface of females, we predicted and later

certified through synthesis that 3,11-dimethylheptacosan-2-one (4) also serves as a sex

pheromone component in this cockroach (Eliyahu et al., 2004; Jurenka et al., 1989;

Schal et al., 1990). This component is less abundant on the female’s cuticular surface

and its behavioral activity is significantly lower in dose-response studies than that of its

C\textsubscript{29} homolog.

Two much less abundant sex pheromone components have also been identified:
the alcohol 29-hydroxy-3,11-dimethylnonacosan-2-one (2) and the aldehyde 29-oxo-

\[ \text{3,11-dimethylnonacosan-2-one (3); interestingly, the alcohol has been shown to be} \]

about 10-fold more behaviorally active than the C\textsubscript{29}-dimethyl ketone (reviewed by

Nishida and Fukami, 1983). The biochemical pathway that gives rise to the alcohol and

aldehyde components has not been elucidated, but Chase et al. (1992) proposed that

similar female-specific hydroxylation and subsequent oxidation reactions that result in

the dimethyl ketones might act at the C-29-position of the dimethyl ketone to produce

the 29-hydroxy- and 29-oxo-dimethyl ketone pheromone components.

Although this hypothesis has yet to be tested with labeled precursors, it predicts

as well, that if the same mechanism converts 3,11-dimethylheptacosane to the

corresponding dimethyl ketone pheromone, then its two oxidation analogs, 27-hydroxy-

and 27-oxo-3,11-dimethylheptacosan-2-one, might also function as pheromone

components in the female German cockroach. We now report behavioral and analytical

results showing that 27-hydroxy-3,11-dimethylheptacosan-2-one and 27-oxo-3,11-
dimethylheptacosan-2-one (5 and 6, respectively, Fig. 3.1) indeed are found on the cuticular surface of adult females, and we confirm their pheromonal activity by synthesis.

![Chemical structures](image)

**Fig. 3.1** Components of the contact sex pheromone of female *B. germanica*. Compounds 5 and 6, within the box, are identified as new pheromone components in this paper.

**Materials and Methods**

*Insects.* *Blattella germanica* cockroaches were kept in groups at 27°C under a 12:12 light-dark photoperiod with continuous access to dry LabDiet rat chow (#5001; PMI Nutrition International, Brentwood, MO, USA) and water. Nymphs that hatched within 2–3 days were reared in synchronous cohorts and newly emerged adult males and females were separated daily from cages containing late instar nymphs.

*Behavioral Assay.* Male sexual response was tested using a modification of the assay developed by Roth and Willis (1952). An antenna of a 14–21 day-old adult male *B. germanica* was excised, inserted into a small mass of modeling clay at the end of a glass Pasteur pipette, and extracted briefly in hexane to remove male cuticular lipids. A test fraction or standard solution was then applied with a 10 µl syringe (Hamilton, Reno, NV, USA).
USA) in 3 µl hexane to the distal 1 cm of the test antenna. The hexane was allowed to evaporate and the antenna was used immediately to test the responses of at least three groups of 10 males 14–21 days old that were housed individually in 9 x 9 x 7.5-cm plastic cages supplied with rat chow and water. All assays were conducted during mid-scotophase, avoiding the first and last 2 hrs of the scotophase. The antennae of each male were gently stroked with the test antenna, and a positive response was recorded when the male executed a courtship response, rotating his body relative to the stimulus and raising his wings within 30 sec. This is an unmistakable response that occurs only in a sexual context and is never elicited by male test antennae either unfortified with female pheromone or treated with hexane alone.

**Extraction and Fractionation.** Sexually mature females, 6–7 days old, were extracted in groups of 50 females in a 20 ml vial with ~6 ml hexane (Optima; Fisher Scientific, Waltham, MA, USA) for 1 min. The extract was transferred to a clean vial and the hexane was slowly reduced to 100 µl under a gentle stream of high purity N₂. The extract was then subjected to fractionation by flash column chromatography: 14.5 cm disposable borosilicate glass Pasteur pipettes loaded with 200 mg of chromatographic silica gel (100–200 mesh; Fisher Scientific) were activated at 110°C for 30 min and washed with ~1 ml hexane. The extract was then loaded and eluted successively with 4 ml hexane, 2 ml each of 1, 2, 5, 10, 20, and 40% diethyl ether (Optima; Fisher Scientific) in hexane, 2 ml diethyl ether, 2 ml ethyl acetate (Optima; Fisher Scientific), and 2 ml methanol (HPLC grade; Fisher Scientific). Each fraction was tested in the courtship behavioral assay, and active fractions were further fractionated on a normal phase high performance liquid chromatography (HPLC) column (Econosphere silica 250 x 4.6 mm, 5 µm; Alltech, Deerfield, IL, USA) on an HP1050 HPLC (Hewlett-Packard, Palo Alto, CA, USA). Supellapyrone (Charlton et al., 1993) was added as internal standard and monitored at 296 nm with an HP1050 diode array detector; the sex pheromone components have no UV absorption. The sample was eluted isocratically at
1 ml min\(^{-1}\) with 99% hexane and 1% 2-propanol (HPLC grade; Fisher Scientific). One min fractions were collected and tested behaviorally on at least 30 males.

An HP5975 mass selective detector, operated in electron impact ionization mode and coupled to an HP6890 GC (Agilent, Santa Clara, CA, USA), was used for chemical structure determinations in active fractions. The GC was operated in splitless injection mode and fitted with a 30 m x 0.25 mm ID DB-5MS column (Agilent). The oven was programmed from 60\(^0\)C to 300\(^0\)C at 15\(^0\)C min\(^{-1}\) after an initial delay of 2 min, and held at 300\(^0\)C for 20 min. Injector temperature was 280\(^0\)C, MS quad 150\(^0\)C, MS source 230\(^0\)C, and transfer line 250\(^0\)C.

**Microchemical Reactions.** 1,1-\(N,N\)-dimethyl hydrazine (DMH, 98%; Sigma Aldrich, St. Louis, MO, USA) derivatization was used to stabilize the thermally unstable oxo-dimethyl ketones prior to GC-MS analysis. The behaviorally active HPLC fraction was reduced under N\(_2\) to ~50 µl in a conical reaction vial, DMH (5 µl) added, and the vial was incubated in a 60\(^0\)C glass bead bath for 30 min.

Silylation of the hydroxy group was conducted prior to GC-MS analysis of the hydroxy-dimethyl ketones. The behaviorally active 100% ether fraction from flash chromatography of 120 females was reduced under N\(_2\) in a conical reaction vial, \(N\)-methyl-\(N\)-trifluoroacetamide (80 µl MSTFA; Alltech) was added, and the reaction mixture was incubated in an 80\(^0\)C bead bath for 30 min.

**Synthesis of 27-hydroxy- and 27-oxo-3,11-dimethylheptacosan-2-one.** (3\(S\),11\(S\))-27-hydroxy-3,11-dimethylheptacosan-2-one and its oxidation product (3\(S\),11\(S\))-27-oxo-3,11-dimethylheptacosan-2-one were synthesized by K.M. The synthesis was executed based on an improved version of the previous synthesis of (3\(S\),11\(S\))-29-hydroxy-3,11-dimethylnonacosan-2-one (Mori et al., 1981). Oxidation of the alcohol to the aldehyde was carefully carried out with Dess-Martin periodinane to avoid racemization at C-3. Details of the synthesis will be published separately by K.M.
Results

The cuticular extract of sexually mature females was fractionated into three discrete behaviorally active flash chromatography fractions, with activity at 2%, 20% and 100% ether, corresponding to the synthetic pheromone components 3,11-dimethylnonacosan-2-one, 29-oxo-3,11-dimethylnonacosan-2-one, and 29-hydroxy-3,11-dimethylnonacosan-2-one, respectively (Fig. 3.2). The elution pattern of the pheromone components also corresponded to that reported by Nishida and Fukami (1983).

To determine if the 100% ether fraction also contained the predicted 27-hydroxy-3,11-dimethylheptacosan-2-one, this fraction was subjected to silylation prior to analysis by GC-MS. The derivatized fraction contained a major peak with the same retention time on GC as the silylated authentic compound 2, 29-hydroxy-(3S,11S)-dimethylnonacosan-2-one (Fig. 3.3a,b), and the two had identical mass spectra, represented in Fig. 3.3c by the spectrum of the natural product. Another prominent peak in the natural extract eluted 4 min before compound 2, at the same retention time as the silylated authentic compound 5 (Fig. 3.3a,b), and had a molecular ion of 510 (after silylation) and a mass spectrum that corresponded with that of the silylated compound 5, 29-hydroxy-3,11-dimethylheptacosan-2-one. Characteristic ion fragments included m/z 72, indicating the presence of a methyl branch on the carbon adjacent to the carbonyl group, and a high intensity fragment at m/z 495 (M+−15), indicating the silylation of a terminal hydroxy group (Fig. 3.3d).

The amount of 27-hydroxy-3,11-dimethylheptacosan-2-one was estimated by comparison to authentic compound 2 to be ~5.3 ng per female, which is about five-fold less than the amount of 29-hydroxy-3,11-dimethylnonacosan-2-one found on the cuticular surface of sexually receptive females. Each of these alcohols is about 18-fold less abundant than the equivalent chain length dimethyl ketone, from which the alcohol presumably arises (Table 3.1). Preliminary dose-response studies with 27-hydroxy-(3S,11S)-dimethylheptacosan-2-one indicate that when loaded onto an inactive test antenna, by itself it can stimulate males to court at a dose about 10-fold less than the C27 dimethyl ketone (data not shown).
The 20% ether fraction was subjected to further behaviorally-guided fractionation by HPLC. Two discrete behaviorally active fractions were obtained: the first 1-min fraction co-eluted with synthetic compound 3, 29-oxo-3,11-dimethylnonacosan-2-one, while the second fraction eluted several min later (data not shown). To determine if the first fraction also contained the predicted 27-oxo-3,11-dimethylheptacosan-2-one, we derivatized this fraction with DMH and analyzed the reaction products by GC-MS in comparison with similarly derivatized authentic compounds 3 and 6. The derivatized active female fraction contained a peak at the same retention time as derivatized authentic compound 3 (Fig. 3.4a,b) and both gave identical mass spectra, represented in Fig. 3.4c by the mass spectrum of the derivatized natural product.

As predicted, 27-oxo-3,11-dimethylheptacosan-2-one (6) was also found in the same active fraction. Its DMH-derivatized form eluted about 4.5 min earlier than the DMH-derivatized C_{29} homolog, at the same retention time on the GC, and with the same ion fragmentation pattern, as the derivatized authentic compound 6 (27-oxo-(3S,11S)-dimethylheptacosan-2-one). Its molecular weight, with M^{+} of 478 (after DMH derivatization), corresponds with that of compound 6, and it has its diagnostic fragment ions, including at m/z 72 for the methyl branch adjacent to the carbonyl group, and m/z 86 yielded by the McLafferty rearrangement of the derivatized carbonyl group in position C-29 (Fig. 3.4d).

The amount of 27-oxo-3,11-dimethylheptacosan-2-one, estimated by comparison with known amounts of 29-oxo-3,11-dimethylnonacosan-2-one, is ~0.15 ng per female. Thus the ratio of the C_{29} aldehyde to its C_{27} homolog is about 4:1. These oxo-dimethyl ketone components are about 40-fold less abundant than the respective alcohols (compounds 2 and 5, Table 3.1). Preliminary dose-response studies with 27-oxo-(3S,11S)-dimethylheptacosan-2-one confirm that independently it alone can elicit sexual responses in males at doses similar to those required with the C_{27} dimethyl ketone alone (data not shown).
The 20% ether fraction from flash column chromatography also contained a second behaviorally-active fraction that eluted within 1-min (1 ml) in normal phase HPLC, but several min after the 27-oxo- and 29-oxo-dimethyl ketones. It may represent additional sex pheromone components whose composition is currently under investigation.

**Table 3.1** Amounts and ratios of pheromone components on the cuticular surface of adult female *B. germanica*

<table>
<thead>
<tr>
<th>Pheromone component</th>
<th>C$_{27}$ components$^d$</th>
<th>C$_{29}$ components$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount (ng)</td>
<td>Ratio</td>
</tr>
<tr>
<td>Dimethyl ketona</td>
<td>97</td>
<td>100</td>
</tr>
<tr>
<td>Hydroxy-dimethyl ketone$^b$</td>
<td>5.3</td>
<td>5.5</td>
</tr>
<tr>
<td>Oxo-dimethyl ketone$^c$</td>
<td>0.15</td>
<td>0.15</td>
</tr>
</tbody>
</table>

$^a$ 3,11-dimethylheptacosan-2-one and 3,11-dimethylnonacosan-2-one, respectively.
$^b$ 27-hydroxy-3,11-dimethylheptacosan-2-one and 29-hydroxy-3,11-dimethylnonacosan-2-one, respectively.
$^c$ 27-oxo-3,11-dimethylheptacosan-2-one and 29-oxo-3,11-dimethylnonacosan-2-one, respectively.
$^d$ Based on determination by GC; ratios relative to 100% of the respective dimethyl ketone.
Fig. 3.2  Percentage of males (N>90 per fraction) responding to synthetic compounds 1, 2 and 3, 10 ng each (a); and flash column chromatography fractions of female cuticular extract (0.05 female equivalents) (b). Numbers (fractions) between hexane and ether represent % ether in hexane. EtOAc = ethyl acetate; MeOH = methanol.
Fig. 3.3 Two separate total ion chromatograms of 160 ng of the silylated synthetic compound 2, 29-hydroxy-3,11-dimethylnonacosan-2-one, and 100 ng of the silylated synthetic compound 5, 27-hydroxy-3,11-dimethylheptacosan-2-one (a), and the similarly silylated 100% ethyl ether fraction of extract of 120 females fractionated by flash column chromatography (b). (c) and (d) are the electron impact mass spectra of peaks in the female extract with retention times corresponding with those of compounds 2 and 5, respectively.
Fig. 3.4 Two separate total ion chromatograms of 300 ng of the derivatized synthetic compound 3, 29-oxo-3,11-dimethylnonacosan-2-one, and 100 ng of the derivatized synthetic compound 6, 27-oxo-3,11-dimethylheptacosan-2-one (a), and the similarly derivatized active HPLC fraction of the combined 20% ethyl ether fractions from the extract of 120 females fractionated by flash chromatography (b). (c) and (d) are the electron impact mass spectra of peaks in the female extract with retention times that corresponded with those of compounds 3 and 6, respectively. The spectra are enlarged 10-fold after m/z 120.
Discussion

Semiochemicals, including sex pheromones, are generally identified by the well-established approach of behavior-guided sequential fractionation of extracts followed by chemical analysis, structure elucidation, and confirmation of synthetic compounds with behavioral activity. A complementary approach predicts candidate pheromone components based on a thorough understanding of the chemistry and biochemistry of pheromone production, possibly coupled with insight into the evolutionary relationships among taxa. For example, Bjostad et al. (1984) predicted the structures of sex pheromone components of the cabbage looper, *Trichoplusia ni*, based on knowledge of their biosynthetic pathway and fatty acid precursors. The pheromone gland of this noctuid moth utilizes acetate to produce the fatty acids octadecanoate and hexadecanoate which undergo Δ11-desaturation to produce Z11-18:acid and Z11-16:acid. But the major component of the sex pheromone is (Z)-7-dodecen-1-yl acetate (Z7-12:OAc). When labeled Z11-16:acid was applied to glands it incorporated into both Z7-12:acid and the corresponding acetate ester pheromone. Because the main pheromone component is produced through a Δ11-desaturation followed by limited chain shortening, and finally reduction and acetylation to form the acetate ester, Bjostad et al. (1984) reasoned that other pheromone components would be produced from intermediates of the chain shortening reactions. Indeed, other acetate esters including Z9-14:OAc, Z7-14:OAc, and Z5-12:OAc were found to serve as pheromone components, the first being derived from limited chain shortening of Z11-16:acid and the latter two from Z11-18:acid. In a similar manner, pheromone components of the spruce budworm (*Choristoneura fumiferana*) were predicted based on identification of fatty acids in the gland and their temporal variation in relation to pheromone production (Silk and Kuenen, 1986). Nonetheless, few examples have been reported of the candidate pheromone approach from predictions based on biosynthetic pathways. Here we confirm that candidate compounds, predicted based on the biosynthetic pathway of related contact sex pheromone components, do indeed serve as pheromone components in the German cockroach.
In insects, cuticular hydrocarbons and hydrocarbon pheromones are formed through fatty acid elongation followed by decarboxylation (review: Howard and Blomquist, 2005). Thus, in the housefly, the sex pheromone component Z9-tricosene is first formed, and this alkene, in turn, is metabolized by a cytochrome P450 enzyme to the corresponding epoxide and ketone that also serve as sex pheromone components (review: Blomquist, 2003). This conversion of hydrocarbons to methyl ketone and epoxide pheromones in the housefly served as the guiding scheme for our research on pheromone biosynthesis in the German cockroach.

The major cuticular hydrocarbon in all life stages of the German cockroach is an isomeric mixture of 3,7-, 3,9- and 3,11-dimethylnonacosane (Jurenka et al., 1989). But the cuticular dimethyl ketone fraction of adult females, which contains the pheromone 3,11-dimethylnonacosan-2-one, comprises only the 3,11-isomer, leading Jurenka et al. (1989) to propose a biosynthetic relationship between the hydrocarbon and its dimethyl ketone pheromone analog. Using radiotracers coupled with radio-GC, Chase et al. (1992) examined the hypothesis that the dimethyl ketone sex pheromone arises from the insertion of an oxygen into the preformed 3,11-dimethyl alkane. Radioactivity from the alkane was detected in both 3,11-dimethylnonacosan-2-ol and 3,11-dimethylnonacosan-2-one, but only in adult females, whereas when tritiated 3,11-dimethylnonacosan-2-ol was applied to the cuticle it was readily and highly efficiently converted to the corresponding dimethyl ketone pheromone not only in females, but also in males. These results confirmed that the dimethyl ketone sex pheromone of *B. germanica* arises via a female-specific hydroxylation of 3,11-dimethylnonacosane and a subsequent non-sex-specific oxidation to the 3,11-dimethylnonacosan-2-one pheromone.

The same mechanism could also convert 3,11-dimethylheptacosane, a component of the cuticular hydrocarbon profile of the German cockroach, to the candidate pheromone component 3,11-dimethylheptacosan-2-one, and indeed, dose-response studies with both natural and synthetic 3,11-dimethylheptacosan-2-one confirmed its behavioral activity, though it was found to be ~10-fold less active than its C_{29} homolog (Schal et al., 1990; Eliyahu et al., 2004).
Chase et al. (1992) also proposed that a similar hydroxylation and subsequent oxidation at the C-29-position of 3,11-dimethylnonacosan-2-one might give rise to 29-hydroxy- and 29-oxo-3,11-dimethylnonacosan-2-one, the other components of the contact sex pheromone blend, and that the same mechanism might convert 3,11-dimethylheptacosan-2-one to its 27-hydroxy- and 27-oxo- analogs.

Identification of the C$_{27}$ alcohol and aldehyde as sex pheromone components is fraught with difficulties, and it is not surprising that earlier studies with cuticular extracts of more than 200,000 females did not reveal these compounds. The two active homologs in each class (dimethyl ketones, alcohols, aldehydes) differ only in chain length, and therefore can only be separated by high performance fractionation methods, such as preparative GC. However, the aldehydes are thermolabile and cannot be subjected to GC analysis without derivatization, and both C$_{27}$ alcohol and aldehyde occur in minute amounts that are largely obscured by the more abundant and presumably more behaviorally active C$_{29}$ homologs. Therefore, predictions based on the biosynthetic pathway of the C$_{29}$ components were crucial for the recognition and subsequent identification of the three C$_{27}$ components.

Our finding of the predicted C$_{27}$ compounds in the cuticular pheromone blend, and in a very similar blend ratio to that of the C$_{29}$ components further corroborates the biosynthetic pathway proposed and partly demonstrated by Chase et al. (1992) (review: Schal et al., 2003). Furthermore, this research now motivates a re-examination of other hydrocarbons that might serve as precursors for behaviorally active methyl ketones and their oxidation derivatives. A prime candidate for consideration is 3,11-dimethylhentriacontane, which occurs on the cuticular surface in tiny amounts (Carlson and Brenner, 1988). Structure-activity studies on 3,11-dimethylnonacosan-2-one indicate that the apparently promiscuous pheromone receptor of B. germanica males accepts chain lengths that are slightly shorter or longer than the optimal 29-carbon alkyl chain (Nishida & Fukami, 1983). Since the C$_{27}$ homolog was found to be behaviorally active, we predict that the C$_{31}$ homolog will show behavioral activity as well.
Although the 3,11-dimethyl configuration is important for behavioral activity, structure-activity studies have shown that both the 3-monomethyl and 11-monomethyl configuration also can elicit sexual responses, albeit at much lower doses (Nishida and Fukami, 1983). The epicuticular surface of B. germanica contains 11-methylheptacosane, 11-methylnonacosane, 11-methyltriacontane, 3-methylheptacosane, 3-methylnonacosane, as well as dimethyl alkanes that include an 11-methyl branch, including 11,15-dimethylheptacosane, 11,15-dimethylnonacosane, 5,11-dimethylheptacosane, and 5,11-dimethylnonacosane (Augustynowicz et al., 1987; Carlson and Brenner, 1988; Jurenka et al., 1989). It is possible that the same mechanism that inserts a C-2 carbonyl into the preformed dimethyl alkane might do the same with a 3-methyl or 11-methyl alkane of the proper chain length. We recently discovered that oxidation metabolites of 11-methylheptacosane are indeed behaviorally active. Nishida and Fukami (1983) found that the antennae of the Oriental cockroach, Blatta orientalis, when stroked against the antennae of male German cockroaches elicit courtship in the latter species. Through extensive fractionation and behavioral assays we recently identified two behaviorally active compounds: 11-methylheptacosan-2-one and 27-oxo-11-methylheptacosan-2-one (Eliyahu et al., unpublished [chapter 5]). The hydrocarbon 11-methylheptacosane is abundant on the cuticular surface of the Oriental cockroach (Lockey and Dularay, 1986), and it is likely that in this cockroach too, the methyl ketone and aldehyde are oxidation products of the hydrocarbon. Again, 3-methyl and 11-methyl hydrocarbons of the appropriate chain length are present in the cuticular lipids of German cockroach females, and they similarly could be converted to behaviorally active methyl ketones, alcohols and aldehydes.

Based on the structure-activity studies conducted on the C_{29} components (Nishida and Fukami, 1983), we predict that the C_{27} alcohol and aldehyde, compounds 5 and 6, likewise will be more behaviorally active than their C_{27} parent compound 4. We are currently conducting dose-response studies to test this hypothesis. Since there are no synergistic effects among the C_{29} dimethyl ketone and alcohol, nor with the C_{27} dimethyl ketone (Schal et al., 1990), it appears that the total activity might be achieved
by additive effect, with low amounts of the more polar compounds being compensated by their higher activity. Thus, even small amounts of individually active components can elicit courtship in adult males when perceived in a blend.

Further studies are necessary to determine the definitive number of components in the contact sex pheromone blend of the female German cockroach. Two important points of caution are worth mentioning: First, the sex pheromone reception system of the German cockroach male exhibits a highly promiscuous response, accommodating compounds that deviate significantly from the native pheromone. Indeed, it represents one of few cases where the native pheromone can be significantly improved upon, for example by changing the C-2 carbonyl to a C-2 hydroxy group. This might explain in part frequent observations of interspecific courtship in cockroaches, as detailed above. But it highlights the important point that for a pheromone component to be considered as such, it must not only elicit the sexual response in males, but it also must be present on the female.

Second, the relative behavioral value to the male of each female pheromone component must be considered. The dimethyl ketone is generally represented on the cuticular surface at 10% of the abundance of its precursor hydrocarbon analog (Chase et al., 1992), and the alcohol and aldehyde occur at 18-fold and 600-900-fold lower amounts than their respective parent dimethyl ketone (Table 3.1). Assuming that other, yet to be identified, behaviorally active components occur at similar ratios, it is apparent that if they arise from relatively poorly represented hydrocarbons, they might occur at sub-nanogram amounts. Moreover, these compounds are presumably spread over the total cuticular surface, and possibly buried within hundreds of micrograms of cuticular hydrocarbons. It remains to be determined how much of each component is “operationally functional”, that is, perceived by the male when he briefly strokes the female’s antennae. We previously determined that each female antenna contains about 1 ng 3,11-dimethylnonacosan-2-one and 0.4 ng 3,11-dimethylheptacosan-2-one, matching closely the minimal amounts required to elicit courtship behavior in males, as determined from dose-response studies (Eliyahu et al., 2004). It is conceivable that
many minor pheromone components are represented on the cuticle, and particularly on the female antennae, at amounts substantially below the male’s threshold of detection, and thus they may represent “noise” arising from an efficient biochemical process rather than serving as mediators of sexual behavior.

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References


CHAPTER 4

German cockroach (Blattella germanica) nymphs elicit courtship in males:

Precocious sexual maturation or sexual mimicry?

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This chapter consists of a manuscript that will be submitted for publication in Animal Behaviour
The male German cockroach performs a characteristic courtship behavior upon contacting a sexually receptive female: He turns away from the female and raises his wings, thereby exposing tergal glands with attractive and nutritious secretions. The female then mounts the male and feeds upon these pre-nuptial secretions; this behavior places her in the appropriate pre-copulatory position. A contact sex pheromone on the cuticular surface of the female, responsible for eliciting courtship behavior in males, consists of a blend of six components that share a common biosynthetic origin. We found that antennae taken from either male or female nymphs of various ages also elicit the full courtship response in adult males. We extracted behaviorally active compounds from the cuticular surface of nymphs and, guided by behavioral assays, we fractionated the extracts using various preparative chromatography procedures, including flash (column) chromatography, high performance liquid chromatography and gas chromatography. Mass spectrometry of chemically-derivatized compounds revealed two classes of courtship-eliciting compounds: All nymphs possess a novel compound that elicits courtship in adult males. In addition, last instar females contained four of the six adult female-specific contact sex pheromone components, consistent with dimorphic differentiation of the sexes at this stage and early sexual maturation of the pheromone biosynthetic machinery. Our results suggest that nymphs might engage in sexual mimicry to avoid cannibalism or to deceitfully gain access to male-produced pre-nuptial tergal secretions which can be accessed only during courtship.
INTRODUCTION

Courtship behavior is often a resource-exhausting activity, yet it is crucial for sexual reproductive success. Species- and sex-recognition signals are important in targeting courtship toward potentially suitable mates, thereby allocating resources efficiently while minimizing risks of predation and parasitism. Qualitatively and quantitatively unique sex pheromone blends are exceptionally effective species-specific signals for discriminating between the sexes and in most cases they unambiguously specify the reproductive state of a potential mate, thus facilitating the recognition of receptive conspecifics of the opposite sex (Ringo 1996; Roelofs 1995; Rafaeli 2002).

Nevertheless, sex pheromones, like other sexual signals, can be exploited by predators and parasitoids that use them to locate prey or hosts, or emit mimetic pheromone analogs to attract prey (Haynes & Yeargan 1999).

In the German cockroach a complex courtship repertoire is vital for mating. The sexes are brought together by means of a volatile pheromone emitted only by receptive females that display a typical calling behavior (Liang & Schal 1993; Nojima et al. 2005) Upon contact of the male antennae with the female’s cuticle the male displays a characteristic courtship: He rotates 180° away from the female and raises his wings, thereby exposing specialized glands that serve as nutrient reservoirs on the 7th and 8th tergites, and directs these glands toward the female’s antennae and gustatory organs. The secretions of these glands – proteins, lipids and especially sugars – serve as attractants and phagostimulants. When the female mounts the male to feed on the secretion, she is placed in an appropriate position for copulation (Nojima et al. 1999; Roth & Willis 1952).

Courtship behavior is elicited by a blend of six contact sex pheromone components identified as oxidation derivatives of cuticular long-chain dimethyl alkanes. The most abundant component is the dimethyl ketone 3,11-dimethylnonacosan-2-one (1, Fig. 4.1), and less abundant components include its derivatives 29-hydroxy-3,11-dimethylnonacosan-2-one (2), and 29-oxo-3,11-dimethylnonacosan-2-one (3).
Additional components are the homologous C_{27} compounds, including 3,11-dimethylheptacosan-2-one (4) and its oxidation derivatives 27-hydroxy-3,11-dimethylheptacosan-2-one (5), and 27-oxo-3,11-dimethylheptacosan-2-one (6); the C_{27} homologues were predicted based on the biosynthetic pathway (Nishida & Fukami 1983; Schal et al. 1990b) and we recently identified components 5 and 6 in extracts of virgin females (Eliyahu et al., unpublished [chapter 3]). Contact sex pheromone production in the German cockroach is regulated by juvenile hormone (JH) which also paces and controls yolk protein synthesis and oocyte maturation. The adult female produces large quantities of pheromone when she becomes sexually receptive approximately 4–6 days after eclosion. Production of the pheromone diminishes dramatically after the female oviposits and remains low during a three-week gestation while she incubates an egg case (Schal et al. 1990a; Schal et al. 1991). Thus, female sex pheromone production in the German cockroach is related to differentiation of pheromone producing cells (oenocytes) and hormonal regulation of dimethyl ketone production (Fan et al. 2003).

Interestingly, courtship in adult males can also be elicited by either male or female teneral (newly eclosed) adults, some heterospecific cockroaches, and even by unrelated insects (Nishida & Fukami 1983). It is not known whether teneral adults employ the same contact sex pheromone components that receptive females use to stimulate courtship in males. Although teneral females produce a small amount of the contact pheromone, teneral males do not (Schal et al. 1990a). Teneral nymphs also elicit sexual responses in males, and it was suggested that "moulting fluids contain a stimulating substance" (Roth & Willis 1952), but the identity of these compounds remains unknown.

We observed that older female nymphs, several days past the molt, retained the capacity to elicit courtship responses in adult males (Fig. 4.2). We suspected that last instar females, which have begun to undergo sexual differentiation, might produce small, but behaviorally sufficient amounts of one or more of the female contact sex pheromone components. Sexual dimorphism of several morphological and physiological features is
quite evident in last instars, including body size and shape, cell numbers of the corpora allata (CA) which produce JH (Chiang et al. 1991), production of JH and its release into the hemolymph by females toward the end of the last instar (Treiblmayr et al. 2006), and differentiation of the fat body so that upon induction with JH, the fat body of last instar females can synthesize small yet significant amounts of vitellogenin, an adult female-specific yolk protein (Kunkel 1981). We therefore hypothesized that precocious differentiation of the pheromone-producing machinery in last instar females might render them stimulatory to adult males. Consistent with this hypothesis, we predicted that last instar males and young nymphs of both sexes would not possess the adult female-specific sex pheromone components, and therefore would fail to elicit courtship in males.

We conducted experiments to test these hypotheses by monitoring male sexual responses to the ontogenetic changes that occur in male and female nymphs throughout nymphal development. We also conducted qualitative and quantitative comparisons of nymph extracts of both sexes with the extract of mature adult females to determine if any of the active compounds on the nymph are similar to the known components of the female contact sex pheromone.

**Figure 4.1.** Components of the contact sex pheromone of female *B. germanica*. Compounds 5 and 6 based on Eliyahu et al. (chapter 3).
Figure 4.2. An adult male *B. germanica* exhibits courtship toward a female nymph. a) The male antennates the female; b) the male raises his wings and exposes a specialized tergal gland in response to contact with the nymph; c) the nymph mounts the male and feeds on the tergal secretion.
MATERIALS AND METHODS

Insects
*Blattella germanica* cockroaches were kept in groups at 27°C under 12:12 light-dark photoperiod and provisioned dry Purina rat chow and water. First instar nymphs were removed from adult mating cages into a collective nymph rearing container. Third instar nymphs were sexed and caged separately prior to behavioral assays.

Behavioral Assay
Male behavioral response was tested using a modification of the assay developed by Roth and Willis (1952). For testing behavioral activity of fractions from nymph and female solvent extracts, an antenna of a 14–21 day-old adult male *B. germanica* was excised, attached to a glass Pasteur pipette, and extracted briefly in hexane to remove male cuticular lipids prior to application of the test fraction; hexane-extracted male antennae fail to elicit courtship in adult males. A 3 µl hexane solution of a test fraction was then applied to the distal 1 cm of the test antenna. The hexane was allowed to evaporate and the antenna was used immediately to test the responses of several groups of 10 males 14–21 days old that were housed individually in 9 x 9 x 7.5 cm plastic cages supplied with rat chow and water. A similar procedure was conducted using non-treated antennae excised from male and female individuals throughout their nymphal and adult development, to test their capacity to stimulate courtship. All assays were conducted during mid-scotophase, avoiding the first and last two hrs of the scotophase. A positive response was recorded when the male executed a courtship response, rotating his body relative to the stimulus and raising his wings within 30 sec (Fig. 4.3). This is an unmistakable response that occurs only in a sexual context and is never elicited by male test antennae unfortified with female pheromone or extracted with hexane.
Extraction and Fractionation

Nymphs of various ages were separated by sex and extracted in groups of 100 in a 20 ml vial with ~6 ml hexane for 1 min. The extract was removed to a new vial and dried under a gentle stream of high purity N\textsubscript{2} to ~100 µl. The extract was then subjected to fractionation by flash chromatography: disposable borosilicate glass Pasteur pipettes with 200 mg of chromatographic silica gel (100–200 mesh, Fisher Scientific, NJ) were activated at 110°C for 30 min and washed with ~1 ml hexane (optima, Fisher Scientific) prior to application of the extract. The extract was eluted with 4 ml hexane, 2 ml of different percentages of diethyl ether (Fisher Scientific) in hexane (1, 2, 5, 10, 20, and 40\% diethyl ether) and 2 ml diethyl ether, 2 ml ethyl acetate (optima, Fisher Scientific) and 2 ml methanol (HPLC grade, Fisher Scientific). Each chromatographic fraction was tested with the behavioral assay on at least 30 males.

Behaviorally active fractions were subjected to further fractionation, with preparative high performance liquid chromatography (HPLC). In normal phase HPLC (Partisil silica column, 250 x 4.6 mm, 5 µm) the sample was eluted at 1 ml min\textsuperscript{-1} with a constant mix of 99\% hexane and 1\% 2-propanol (both HPLC grade, Fisher Scientific). One-min fractions (1 ml) were collected, gently blown down to dryness, resuspended in hexane, and behaviorally tested on at least 30 adult males. An internal standard, supellapyrone (Charlton et al., 1993), was used to clearly delineate the retention times of fractions.

Chemical Analysis

An HP6890 GC coupled to an HP5975 mass selective detector (Agilent, Palo Alto, CA) were used to identify chemical structures in active fractions. The GC was operated with splitless injection and fitted with a 30 m x 0.25 mm ID DB-5MS column (Agilent). The oven was programmed from 60\textdegree C to 300\textdegree C at 15\textdegree C min\textsuperscript{-1} after an initial delay of 2 min, and held at 300\textdegree C for 20 min. Injector temperature was 280\textdegree C, MS quad 150\textdegree C, MS source 230\textdegree C, and transfer line 250\textdegree C.
Oxo-dimethyl ketones were derivatized with 1,1-\(N,N\)-dimethylhydrazine (DMH, 98%, Sigma-Aldrich, St. Louis, MO) for added thermal stability prior to GC-MS analysis. Behaviorally active HPLC fractions were dried under \(N_2\) to \(~50\) \(\mu\)l in a conical glass reaction vial and 5 \(\mu\)l DMH were added. The vial was incubated in a 60\(^{\circ}\)C glass bead bath for 30 min.

Sexually mature adult females were similarly extracted and their extract fractionated and treated in a similar manner for comparison.

Figure 4.3. The behavioral assay used for testing activity of fractions: A test antenna is attached to a glass Pasteur pipette and gently stroked against the antennae of individually caged males (a); An isolated test antenna treated with courtship-eliciting compounds stimulates courtship in males.
RESULTS

1. **Nymphs elicit courtship throughout their development**
Antennae taken from nymphs of various ages, both males and females, elicited courtship responses in 80–100% of adult males (Fig. 4.4). As expected, teneral adults also elicited courtship responses, especially when still un-sclerotized. The capacity of female antennae to stimulate courtship diminished noticeably with age, only to increase again to a maximum level as they reached sexual maturity 6 days after eclosion. Adult males completely lost the capacity to elicit courtship 2 days after emergence (Fig. 4.4).

2. **Courtship eliciting components in nymphs**
Figure 4.5 depicts the activity of extracts of adult females and nymphs following fractionation by flash chromatography. Three fractions were consistently recovered from hexane extracts of adult females: The 2% diethyl ether fraction contained the C$_{27}$ and C$_{29}$ dimethyl ketones (1 and 4), while the 20% and 100% diethyl ether fractions contained the 27- and 29-oxo-dimethyl ketones (3 and 6) and 27- and 29-hydroxy-dimethyl ketones (2 and 5), respectively (Fig. 4.5a). Two active fractions were recovered from the extracts of last instar females, corresponding to the dimethyl ketone fraction and to the oxo-dimethyl ketone fraction (10% and 20% diethyl ether). Extracts of last instar male nymphs, on the other hand, contained only a single active fraction, coeluting with oxo-dimethyl ketones of the adult female (Fig. 4.5b). Extracts of early instar female and male nymphs contained the same behaviorally active fraction at 20% ether as last instar males.

2.1. **Courtship eliciting compounds in last instar female nymphs**
The behaviorally active 2% ether fraction from last instar females was further fractionated by HPLC (data not shown). Two dimethyl ketones, identical to those of the adult female (1 and 4), were found in the active HPLC fraction when analyzed by GC-MS and compared to synthetic dimethyl ketone standards (Fig. 4.6). These compounds
have characteristic high molecular ions (M+ = 450 and 422, respectively) and an ion fragment at m/z 72 indicative of a 2-keto group with an adjacent methyl branch in the hydrocarbon chain. Ion fragments at m/z 127 and 197 indicate a second methyl branch on C-11, and reduction of these ketones to the respective hydrocarbons confirmed that only the 3,11-positional isomers of the hydrocarbon were present (data not shown). Using an internal standard (14-heptacosanone), the amounts of 3,11-dimethylheptacosan-2-one and 3,11-dimethylnonacosan-2-one were estimated at 2 and 4 ng per female nymph, respectively. This corresponds well with behavioral dose-response studies showing that ~1 ng 3,11-dimethylnonacosan-2-one elicits responses in 50% of males, and this response increases sharply to 100% at ~2 ng (Eliyahu et al. 2004).

The behaviorally active 10% and 20% ether fractions of both adult females and last instar female nymphs were also fractionated further using preparative HPLC. The 20% ether fraction of adult females was further separated into two active fractions by HPLC, one coeluting with the oxo-dimethyl ketones (3 and 6) 1 min before the supellapyrone internal standard, and a new unidentified pheromone component that eluted 2 min after the internal standard (Fig. 4.7). Last instar females contained the same two active HPLC fractions as in the adult female.

The female nymph fraction corresponding to the oxo-dimethyl ketones (1 min before the internal standard; Fig. 4.7) was analyzed in selected ion monitoring (SIM) mode on the GC-MS following derivatization with DMH. The retention times and mass spectra of the DMH products were also compared to those of the derivatized female oxo-dimethyl ketones (Fig. 4.8). Both 27-oxo-3,11-dimethylheptacosan-2-one (6) and 29-oxo-3,11-dimethylnonacosan-2-one (3) were detected in extracts of adult females and last instar female nymphs. Like the dimethyl ketones, these derivatized compounds have high molecular ions (M+ = 464 and 436, respectively; 506 and 478 after derivatization with DMH), ion fragments at m/z 72 indicative of a 2-keto group with an adjacent 3-methyl branch, and m/z 86, indicative of a McLafferty rearrangement of the derivatized aldehyde, and a prominent peak at m/z 127 suggests another methyl branch
in position 11 of the hydrocarbon chain. These results demonstrate that last instar female
nymphs contain both C<sub>27</sub> and C<sub>29</sub> homologs of the aldehyde pheromone, as do adult
females.

2.2. Courtship eliciting compounds in young nymphs and in last instar male nymphs
The behaviorally active flash chromatography fractions from extracts of last instar
males (10% and 20% diethyl ether, Fig. 4.5) were combined and further fractionated by
normal-phase HPLC. A single behaviorally active fraction eluted 2 min after the internal
standard, at the same retention time as the newly discovered courtship-eliciting
compound found in last instar and adult females (Fig. 4.7). This fraction was also active
in extracts of younger male and female nymphs (Fig. 4.7), showing that all life stages of
the German cockroach possess one or more yet to be identified courtship-stimulating
compounds contained in this 1 min (= 1 ml) HPLC fraction. Structural elucidation of
this “universal courtship elicitor” is underway.

![Graph showing percentage of adult males responding to test antennae taken from males and females of various stages in development.](image)

**Figure 4.4.** Percentage of adult males (N=30 per data point) responding to
test antennae taken from males and females of various stages in
development. NE is newly emerged within 6 hrs of the molt; “early”
represents a feeding nymph, whereas “late” represents a non-feeding pre-
molt nymph.
Figure 4.5. Percentage of males (N>90 per fraction) responding to flash chromatography fractions of extracts from females and males. (a): Fractions from adult females were assayed at 0.05 female-equivalents, whereas fractions from last instar female nymphs and early instar female nymphs were tested at 0.6 and 1.0 nymph equivalents, respectively. (b) Fractions from adult males, last instar males, and early instar males were assayed at 0.5, 0.6, and 1.0 insect equivalents. Numbers (fractions) between hexane and ether represent % ether in hexane. EtOAc = ethyl acetate; MeOH = methanol.
**Figure 4.6.** Total ion chromatogram of 100 ng of the synthetic 3,11-dimethyl ketones 1 and 4 (a) and the 2% ethyl ether fraction of extract of 100 last instar female nymphs fractionated by flash chromatography (b). (c) and (d) are the electron ionization mass spectra of peaks in the nymph extract corresponding with the authentic 4 and 1, respectively.
Figure 4.7. Percentage of adult males (N>90 per fraction) responding to HPLC fractions of the combined flash chromatography fractions 10% and 20% ether of females (a) and males (b). Fractions from adult females and were assayed at 0.1 and 0.5 adult insect equivalents, from last instar nymphs were assayed at 0.6 insect equivalents, and fractions from early instar nymphs were tested at 1 nymph equivalents. Time 0 represents the retention time of supellapyrone, which was used as an internal standard, showing that active fractions were consistently recovered 1 min before and 2 min after the internal standard.
Figure 4.8. Mass spectra of two compounds recovered from the HPLC fraction eluting 1 min before the internal standard (see Fig. 7). A cuticular extract of 200 last instar female nymphs was fractionated by flash chromatography, the 10% and 20% ether fractions were combined and fractionated by normal phase HPLC. The behaviorally active 1 min (= 1 ml) fraction was derivatized with DMH, resulting in the mass spectra (a) corresponding with authentic compound 3 and (b) corresponding with authentic compound 6.
DISCUSSION

The phenomenon of immatures eliciting courtship in the German cockroach was first observed by Roth and Willis (1952) who found that 76% and 84% of males tested courted teneral male and female nymphs, respectively; 58% of the males also courted very young adult females, 5 minutes to 4 hrs old. Males were also stimulated to court isolated antennae taken from newly emerged males. Roth and Willis (1952) speculated that the stimulating substance from immatures might be different from that present in sexually mature females and hypothesized that the newly emerged adults, especially males, might acquire it from their shed nymphal cuticles and gradually lose it as they matured.

Based on the gradual reproductive maturation and sexual differentiation in last instar nymphs, we hypothesized that the courtship-eliciting compounds of adult females would be restricted to last instar females of the German cockroach; we also expected all other stages, including young nymphs and adult males, to fail to stimulate courtship in adult males. Our results support the first hypothesis, but provide evidence to reject the second. We found that last instar female nymphs indeed produced at least four of the six sex pheromone components found on adult females, consistent with a gradual onset of an adult female phenotype. Surprisingly, however, all nymphal stages can elicit sexual responses in adult males with varying blends of courtship-inducing compounds.

**Adult female sex pheromone components in last instar females**

Equipped with the elucidated chemical structures of six metabolically related pheromone components, a highly sensitive and discriminating behavioral assay, and finely-tuned chemical analysis instrumentation, we were able to show that last instar female nymphs produce four of the six contact sex pheromone components of sexually mature females, albeit at much smaller quantities. This is in agreement with previous observations that various compounds and processes related to sexually dimorphic maturation begin to be expressed in last instar female nymphs, gradually progressing
toward the sex-specific adult phenotype (Chiang et al. 1991; Engelmann 1990; Kunkel 1981; Treiblmayr et al. 2006).

Using analytical approaches, we could not detect any of the 27- and 29-hydroxy-dimethyl ketones (compounds 2 and 5), which presumably serve as metabolic precursors of the oxo-dimethyl ketones (Schal et al. 1993). Behavioral activity of the 100% ether fraction, which contains these hydroxy-dimethyl ketones of the adult female, was detectable, though rather inconsistently, in extracts of adult females, but not in any nymphs. This inconsistency might be due to the presence of fatty acids in this fraction which, even at relatively low concentrations, have been shown to mask the behavioral activity of 3,11-dimethylnonacosan-2-one (Nishida & Fukami 1983). Nymphs might have proportionally more fatty acids than adult females.

The behavioral activity of the HPLC fraction that contains the two oxo-dimethyl ketones was as high as that of the fraction containing the two dimethyl ketones. Yet the aldehydes were represented in much smaller amounts in both last instars and adult females than were the dimethyl ketones (Eliyahu et al. unpublished [chapter 3]). These results might be explained by several observations. First, the oxo-dimethyl ketones are rather unstable and readily decompose even at ambient temperature during HPLC fractionation and solvent removal, and therefore are less represented in analytical procedures. Second, the 1 min HPLC fraction that contains these aldehydes might also contain related, yet unidentified compounds that contribute to its overall courtship-stimulating bioactivity. We know, for example, that the cuticular surface of the cockroach *Blatta orientalis* contains 27-oxo-11-methylheptacosan-2-one that can elicit courtship in *B. germanica* males (Eliyahu et al. unpublished [chapter 5]). The cuticular surface of nymphs and adults of the German cockroach contains both 11-methylheptacosane and 11-methyleneicosane (Jurenka et al. 1989) that can be metabolized to the respective alcohol and aldehyde analogues.

It is important to mention that in conducting these experiments we adopted special precautions to avoid contamination. We used nymphs that had no contact with adults since hatching, and nymphs were sexed and separated before the molt to the 4th
instar. Also, every GC-MS analysis of nymph extract was preceded by a blank run. Hence, the sex pheromone analogs found in last instar female nymphs could not have originated from contamination from adult females.

**Courtship eliciting compounds from young nymphs and last instar male nymphs**

Last instar male nymphs and younger nymphs of both sexes do not produce any of the contact sex pheromone components identified in last instar and adult females. Rather, they possess novel, yet to be identified courtship-eliciting compound(s) with similar chromatographic characteristics to those found in adult females and teneral individuals (Eliyahu et al., unpublished). These results indicate that adult females have at least one more sex pheromone component that remains to be identified, and that this compound appears to serve as a “universal courtship elicitor” in this species.

**Why engage in intraspecific sexual mimicry?**

The adaptive value to immature *B. germanica* of stimulating courtship in adult males is not clear, but we propose several reasons based on the natural history of this cockroach and similar cases in other systems. First, nymphs may gain nutritional benefits from mimicking females. Often courted nymphs feed on the tergal secretion of the courting male (Fig. 4.2c), and males appear not to discriminate between a mounting adult female and a nymph. The tergal gland secretion is composed of sugars (maltose and other oligosaccharides) in relatively large amounts (Nojima et al. 2002; Nojima et al. 1999) in addition to proteins, phospholipids, fatty acids and hydrocarbons (Kugimiya et al. 2002). It is important to note that the male’s wings normally conceal the tergal secretion, which becomes accessible only during courtship. Receptive females appear to make mate choices based upon sampling this secretion (Schal, unpublished), suggesting a strong selection pressure on males to forage and stockpile sufficient secretion in their large tergal reservoirs. Nymphs, which are much smaller than adult females, could accrue significant benefit from deceiving males to offer them these pre-nuptial gifts. This sexual deception, and the male’s ready acceptance of immatures as potential mates,
could be further reinforced by the male-biased operational sex ratio in this species; adult females incubate the egg case for about 20 days and are sexually receptive for only 2–3 days during each 25-day ovarian cycle. Moreover, females store sperm and may remain completely unreceptive for several consecutive reproductive cycles, further exacerbating the male-biased operational sex ratio. Hence, the chance of obtaining a mate is low for most males, and males might have evolved a bet-hedging strategy based on relatively non-discriminating courtship of members of its aggregation.

Second, stimulating males to court might also protect nymphs from intraspecific aggression and cannibalism. In the staphylinid beetle, *Aleochara curtula*, immature adult males protect themselves from high levels of intrasexual aggression by producing the female pheromone (Peschke 1985). Also, many arthropod species, especially under conditions of high density and scarceness of food, nibble on body parts of vulnerable teneral individuals. A few examples are the assassin bug *Triatoma rubrofasciata* (Cortéz & Gonçalves 1998), the spiny lobster *Panulirus japonicus* (Matsuda et al. 2003), the spider *Amaurobius ferox* (Kim 2001), the corn earworm *Helicoverpa zea* (Dial & Adler 1990), locusts and cockroaches (*Schistocerca gregaria* and *Blaberus atropos*, personal observations). In the lobster cockroach, *Nauphoeta cinerea*, adult males engage in high levels of intrasexual aggression that can even escalate to killing of opponents. But highly aggressive territorial males also exhibit courtship responses upon contact with teneral individuals, both nymphs and adults (Schal & Bell 1983), suggesting that intraspecific sexual mimicry evolved in these vulnerable individuals to avert potentially fatal agonistic interactions. Moreover, at low density *N. cinerea* males are highly territorial, and by deflecting aggression and stimulating courtship nymphs may gain access to territorial resources. It is therefore possible that newly emerged *B. germanica*, both nymphs and adults, also use sexual mimicry to deflect aggression and cannibalism at this vulnerable stage.

Sclerotized nymphs of the German cockroach, on the other hand, are less vulnerable to damage from adult males. Under normal conditions in the laboratory, German cockroach males are relatively docile compared to gravid females (Breed et al. 1979).
1975). Nevertheless, adult males experience strong selection pressure to forage and procure proteins as part of a paternal investment strategy, and when food is scarce small nymphs may be vulnerable. Toward the conclusion of copulation, adult males transfer to females a urate plug that serves as a paternal nutritional investment in the offspring (Mullins et al. 1992). Females can metabolize this post-nuptial gift and incorporate its constituent nitrogen into amino acids. Moreover, females deficient in nitrogen incorporate more male-derived products into their offspring, indicating that the male’s contribution may play a significant role in offspring fitness (Mullins et al. 1992). A similar situation occurs in another blattellid, *Xestoblatta hamata* (Schal & Bell 1982), suggesting that in cockroach taxa whose adult males possess well-developed uricose glands, including *Blattella*, there might be a premium on protein foraging or urate sequestration by adult males. Males with urate-laden accessory reproductive glands also are under strong pressure to mate because copulation is the only means to void potentially toxic urates. Therefore it might not be difficult to elicit courtship behavior from protein-rich males with a relatively low threshold for both mating and urate excretion, while eliciting courtship in aggressive protein-seeking males might protect nymphs when food resources are scarce.

A third proposal is that the omnipresent courtship eliciting compound(s) found in all of the life stages we tested serve a non-communicative function in the immature stages and are later used in adults as part of the sex pheromone blend. An example for such a case is the Southern masked chafer, *Cyclocephala lurida*. The grubs produce a chemical that renders them attractive to adult males. However, grubs live underground, feeding on roots, while adults are active above-ground with no contact between these life stages. Thus there is no apparent adaptive significance for this sexual mimicry. It has been speculated that the female sex pheromone evolved from the larval odor, which males lose during eclosion and sexual maturation, but females retain (Haynes et al. 1992). Because *B. germanica* live in mixed stage aggregations with overlapping generations, and not in separate niches, we consider this conjecture less probable in this system.
Further studies are needed to illuminate the chemical cues that stimulate courtship of immatures in the German cockroach, the functions of this behavior, and its adaptive and detrimental values to the courted nymphs and the courting male, respectively. How common is this phenomenon in nature? To what extent can males afford to give away their tergal secretion? How effective is deceitful stimulation of courtship at preventing aggression? More generally, how common is this phenomenon of courtship of immatures among cockroaches and related insects? Courtship is the first step in the reproductive process, and a better understanding of the former is needed to fully comprehend the latter.

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CHAPTER 5

Identification of cuticular compounds responsible for eliciting interspecific courtship behavior in the male German cockroach, *Blattella germanica*

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This chapter consists of a manuscript that will be submitted to *Naturwissenschaften*
**Abstract**  Sexual communication is vital for reproductive success in most sexually reproducing species. The cuticular surface of sexually mature females of the German cockroach contains a sex pheromone that, upon contact with the male’s antennae, elicits a distinctive species-specific courtship behavior. This female-specific pheromone consists of a blend of several long-chain methyl ketones, alcohols and aldehydes, all derived from prominent cuticular hydrocarbons that occur in all life-stages of this cockroach. We found that contact with the antennae of 5 out of 20 phylogenetically related as well as unrelated cockroach species elicited courtship behavior in German cockroach males. The heterospecific courtship-eliciting compounds were examined by behaviorally-guided fractionation of the active crude organic extracts and compared to the native sex pheromone components. We identified two active compounds from the cuticular extract of the Oriental cockroach, *Blatta orientalis* – 11-methylheptacosan-2-one and 27-oxo-11-methylheptacosan-2-one – each of which effectively and independently can stimulate courtship in German cockroach males. These compounds share common features with, but are distinct from any of the known contact sex pheromone components. This suggests that the sex pheromone receptor(s) of male German cockroach is unusually promiscuous, accepting a wide range of compounds that share features with its native pheromone, thus resulting in a broad spectrum of behavioral response to heterospecifics. We propose that several features of their mating system—chiefly, absence of closely related species in the anthropogenic environment resulting in relaxation of selection on sexual communication and a highly male-biased operational sex ratio—have driven males to respond with extremely low thresholds to a wide spectrum of related compounds.
**Introduction**

Behavioral reproductive isolation—the species-specific reproductive signals that maximize attraction of conspecifics and minimize attraction of closely related heterospecific individuals—is important in initiating speciation, as well as maintaining it (Coyne and Orr 2004). Qualitative differences in species-specific sex pheromones and quantitative differences in blend ratios can form and reinforce such reproductive behavioral isolation (Löfstedt 1993; Groot et al. 2006). In cockroaches it has been suggested that volatile pheromone blends contribute to reproductive isolation, especially among species of *Periplaneta* and related blattids that use several periplanone pheromone components in different blends (Gemeno and Schal 2004). Few other volatile sex pheromones have been identified in other cockroach species, but two other cockroaches, *Supella longipalpa* and *Blattella germanica*, appear to use single component pheromones (Charlton et al. 1993; Nojima et al. 2005). This is in variance with the typical insect pattern—especially in Lepidoptera—and may be related to minor or no interaction with closely related species, relaxation from such interaction and allopatry brought about by their anthropogenic habits, or possibly lack of concerted efforts by researchers to identify minor constituents of sex pheromones.

The German cockroach, *B. germanica*, an important commensal pest of humans and domestic animals, also uses a highly effective contact pheromone to mediate sexual interactions. The pheromone is produced by sexually-receptive females and it elicits a typical species-specific courtship response in the male; without this pheromone there can be no mating. Upon contact of the male’s antennae with the female’s cuticular surface, the male executes a turn while at the same time raising his wings; he maintains this position momentarily, thereby exposing specialized tergal glands and their nutritious secretion. Sugars and phospholipids in the secretions synergistically serve as phagostimulants (Nojima et al. 1999), and as the female mounts the male to feed on the glandular provisions, she is appropriately positioned for copulation (Roth and Willis 1952).
The contact sex pheromone components are derived from the dimethylalkanes 3,11-dimethylheptacosane and 3,11-dimethylnonacosane, and are listed below from most to least abundant in hexane extracts of the epicuticular surface: 3,11-dimethylnonacosan-2-one, 3,11-dimethylheptacosan-2-one, 29-hydroxy-3,11-dimethylnonacosan-2-one, 27-hydroxy-3,11-dimethylheptacosan-2-one, 29-oxo-3,11-dimethylnonacosan-2-one, and 27-oxo-3,11-dimethylheptacosan-2-one (Nishida and Fukami 1983; Schal et al. 1990; Eliyahu et al. unpublished [chapter 3]). Each of these pheromone components can release courtship independently and there is no evidence for synergism among them (Schal et al. 1990).

Interestingly, Nishida and Fukami (1983) found that unmanipulated isolated antennae from different species of cockroaches and even unrelated insects could elicit courtship in the German cockroach, suggesting that these antennae contain native compounds that can act as contact pheromone mimics. We similarly found in a previous study that male Supella longipalpa antennae can release courtship in German cockroach males (Eliyahu et al. 2004). In light of these findings, we propose two competing hypotheses. First, courtship eliciting compounds on heterospecifics might be identical to one or more contact sex pheromone components of the German cockroach. This scenario could emerge in allopatric species in which common metabolic pathways could give rise to convergent pheromone products. Alternatively, the heterospecific compounds might differ from the native contact sex pheromone components, but retain sufficient common features to act as pheromone analogs. This hypothesis also invokes the notion that the male German cockroach responds to a broad spectrum of pheromone analogs.

To differentiate these two hypotheses, we first tested the capacity of isolated antennae from 20 species of cockroaches to elicit courtship when stroked against the antennae of male German cockroaches. The compounds responsible for releasing this behavior were isolated from the cuticular extract of the Oriental cockroach and identified. We propose evolutionary scenarios and selection forces that might maintain heterospecific sexual signaling.
Materials and methods

Insects  _Blattella germanica_ cockroaches were kept in groups at 27°C under 12:12 light-dark photoperiod with access to dry LabDiet rat chow (#5001; PMI Nutrition International, Brentwood, MO, USA) and water. Newly emerged adult males and females were separated daily from collectively reared nymphs.

The cockroach species listed in Table 5.1 were kept in groups at 27°C under 12:12 light-dark photoperiod and fed dry rat or dog chow and water. Newly emerged adults of species whose antennae elicited courtship response in _B. germanica_ males were separated by sex at eclosion.

Behavioral Assay  Male courtship response was tested using a modification of the assay developed by Roth and Willis (1952). An antenna from different species was excised, attached to a glass Pasteur pipette, and used immediately to test the responses of at least three groups of ten _B. germanica_ males 14–21 d old males that were individually housed in 9 x 9 x 7.5 cm plastic cages. All assays were conducted during mid-scotophase, avoiding the first and last two hrs of the scotophase. A positive response was recorded when the male executed a courtship response, rotating his body relative to the stimulus and raising his wings within 30 sec of being stroked by the test antenna. This is an unmistakable response that occurs only in a sexual context and is never elicited by male test antennae unfortified with female pheromone or treated with hexane alone.

For testing behavioral activity of fractions of cuticular extracts from courtship eliciting species, an antenna of a 14–21 day-old adult male _B. germanica_ was excised, attached to a glass Pasteur pipette, and extracted briefly in hexane to remove male cuticular lipids. A 3-µl hexane solution of a test fraction was then applied with a 10 µl syringe (Hamilton, Reno, NV, USA) to the distal 1 cm of the test antenna. The hexane was allowed to evaporate and the antenna was used immediately in the manner described above.
Extraction and fractionation  Same-sex courtship eliciting individuals were extracted in a 20 ml vial with ~6 ml hexane (Optima; Fisher Scientific, Waltham, MA, USA) for 1 min. The extract was decanted to a clean vial and slowly reduced under a gentle stream of high purity N\textsubscript{2} to ~100 µl. The extract was then subjected to fractionation by flash column chromatography: 14.5 cm disposable borosilicate glass Pasteur pipettes with 200 mg of chromatographic silica-gel (100–200 mesh, Fisher Scientific) were activated at 110°C for 30 min and washed with ~1 ml of hexane prior to application of the extract. The extract was eluted sequentially with 4 ml hexane, 2 ml of each 1, 2, 5, 10, 20, and 40% diethyl ether (Optima; Fisher Scientific), 2 ml diethyl ether, 2 ml ethyl acetate (Optima; Fisher Scientific), and 2 ml methanol (HPLC grade; Fisher Scientific). Each fraction was tested in the courtship assay. Active fractions were further fractionated on a normal phase high performance liquid chromatography (HPLC) column (Econosphere silica 250 x 4.6 mm, 5 µm; Alltech, Deerfield, IL, USA) on an HP1050 HPLC (Hewlett-Packard, Palo Alto, CA, USA). Supellapyrone (Charlton et al. 1993) was added (400 ng) as internal standard and monitored at 296 nm with an HP1050 diode array detector; the native sex pheromone components have no UV absorption. Samples were eluted isocratically at 1 ml min\textsuperscript{-1} with 99% hexane and 1% 2-propanol (HPLC grade; Fisher Scientific). One min fractions were collected and tested behaviorally on at least 30 males.

Preparative gas chromatography (GC)  Behaviorally active HPLC fractions were further fractionated by preparative GC. The HPLC fraction was reduced under N\textsubscript{2} to ~1 µl and injected into a splitless inlet coupled to a non-polar Equity-1 mega-bore column (5 m x 0.53 mm ID, 1.5 µm film thickness; Supelco, Bellefonte, PA, USA) in a modified HP5890II GC. Injector and detector temperatures were set at 280°C, oven temperature increased from 60°C to 300°C by 15°C min\textsuperscript{-1} after an initial delay of 2 min. Detector B was custom modified to accommodate a 20-cm section of mega-bore column onto which eluted compounds were trapped. This column trap could be quickly withdrawn and replaced with a new 20 cm trap. This new preparative GC procedure will
be described separately (Nojima et al. in preparation). Collected GC fractions were eluted from each 20 cm mega-bore column trap with 100 µl hexane into a conical vial for behavioral and chemical analyses.

**Microchemical reactions** 1,1-\(N,N\)-Dimethyl hydrazine (DMH, 98%, Sigma-Aldrich, St. Louis, MO, USA) derivatization was used to stabilize the thermally unstable oxo-methylketones for GC-MS analysis. The behaviorally active HPLC fraction was reduced under \(N_2\) to ~50 µl in a conical reaction vial and 5 µl dimethyl hydrazine were added. The vial was incubated in a 60°C bead bath for 30 min.

A modified Wolff-Kishner reduction was performed on the active preparative GC fraction to determine the methyl branch position: the fraction was combined with 10 µg 14-heptacosanone (as a standard and catalyst) and dried under \(N_2\) in a 0.1 ml conical reaction vial to ~1–2 µl. Twenty µl of 10% hydrazine hydrate in ethanol with trace amount of formic acid were added and the mixture was allowed to stand for ~45 min at room temperature. The solvent was then evaporated and 20 µl of 10% KOH in diethylene glycol were added. The vial was warmed up to 200°C for 30 min, after which its contents were diluted with water and extracted with hexane for analysis by GC-MS.

**Chemical analysis** An HP5975 mass selective detector, operated in electron impact ionization mode and coupled to an HP6890 GC (Agilent, Santa Clara, CA, USA), was used for chemical structure determinations in active fractions. The GC was operated in splitless injection mode and fitted with a 30 m x 0.25 mm ID DB-5MS column (Agilent). The oven was programmed from 60°C to 300°C at 15°C min\(^{-1}\) after an initial delay of 2 min, and held at 300°C for 20 min. Injector temperature was 280°C, MS quad 150°C, MS source 230°C, and transfer line 250°C.
Table 5.1  Species tested for eliciting courtship in adult male German cockroach: antennae, cuticular extract, and flash chromatography fractions of cuticular extract (n=30).

<table>
<thead>
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<th>% males responding to whole antennae of</th>
<th>% males responding to crude extract and its flash chromatography fractions&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>female</td>
<td>male</td>
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<td><strong>BLABERIDAE</strong></td>
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<td>Blaberus atropos</td>
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<td>Blaptica dubia</td>
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<td>Diploptera punctata</td>
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<td>Eublaberus posticus</td>
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<td>Leucophaea maderae</td>
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<td>Schultzia lamprylioides</td>
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<td><strong>BLATTIDAE</strong></td>
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<td>Periplaneta australasia</td>
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<td>P. americana</td>
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<td>P. fuliginosa</td>
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<tr>
<td>Blatta orientalis</td>
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<tr>
<td><strong>BLATTELLIDAE</strong></td>
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<td>Parcoblatta lata</td>
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<td>P. persilobusica</td>
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<td>0</td>
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<tr>
<td>Supella longipalpa</td>
<td>100</td>
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</table>

<sup>a</sup> Extracts of species whose antennae did not elicit response were not tested nor fractionated

<sup>b</sup> Extract of adult females
Results

Courtship by male German cockroach elicited by heterospecific cockroaches  The species tested and the capacity of their isolated antennae – taken from males, females and nymphs – to elicit courtship in German cockroach males, are listed in Table 5.1. Antennae of five of the 20 species tested elicited courtship, showing no obvious phylogenetic pattern because the five species represented all three families. Nevertheless, we did not test other Blattella species and it is likely that such a pattern might emerge at the genus level. In four species, antennae of males, females and nymphs elicited courtship, but Gromphadorhina portentosa male antennae, which are highly sexually dimorphic, did not release courtship.

Sexually mature adult females of the five species were extracted in hexane and behavioral activity of the crude extract assayed. Surprisingly, extract of G. portentosa female failed to release courtship in any of the tested males. The extracts of the other four species were fractionated by flash column chromatography, and behavioral activity of each fraction was tested in the courtship assay (Table 5.1). All four species whose extracts elicited courtship showed activity in the 10 and 20% ether fractions. Two B. germanica pheromone components, the aldehydes 29-oxo-3,11-dimethylnonacosan-2-one and 27-oxo-3,11-dimethylheptacosan-2-one, normally elute in these fractions. Only in Blaptica dubia minor activity was found in the 100% ether fraction, where 29-hydroxy-3,11-dimethylnonacosan-2-one and 27-hydroxy-3,11-dimethylheptacosan-2-one normally elute. Only Blatta orientalis showed activity in the 2% ether fraction, where the two B. germanica dimethly ketone pheromone components, 3,11-dimethylnonacosan-2-one and 3,11-dimethylheptacosan-2-one, normally elute (Eliyahu et al. [chapter 3]).

Chemical identification of the courtship eliciting compounds of B. orientalis  The 2% ether fraction from B. orientalis was further fractionated by normal phase HPLC, yielding a single behaviorally active 1 ml fraction, which was in turn fractionated by preparative GC. Two separate behaviorally active fractions were collected in preparative
GC, a highly active 0.67 min fraction at R_t 23.00–23.67 min and a much less active 0.33 min fraction at R_t 24.00–24.33 min (Fig. 5.1a). The first fraction consisted of a major peak in GC-MS analysis, with M^+ = 408 (Fig. 5.1b) that also constituted the largest peak in the 2% ether fraction (data not shown). MS data suggested a methyl ketone, possibly 11- or 13-methylheptacosanone: m/z 408 indicates the molecular weight of a 27 carbon chain with a single methyl branch, m/z 390 (M^+ -18) indicates the loss of H_2O, a typical fragment in carbonyl-containing compounds.

A C-2 or C-26 carbonyl was suggested by a base peak at m/z 59, but together with a fragment at m/z 127, this pattern suggested an 11-methyl-2-alkanone. To conclusively determine the methyl branch position of the behaviorally active compound, we reduced the active fraction with the Wolff-Kishner reduction (Fig. 5.1c). A new M^+ of 394 and diagnostic fragments at m/z 379 (M^+ -15), 168/169 (C_{12}H_{25}) and 252/253 (C_{18}H_{37}) indicated that the reduction product was 11-methylheptacosane. This assignment remains to be confirmed by comparison of retention time, mass spectrum and behavioral activity with authentic 11-methylheptacosan-2-one.

The combined 10 and 20% ether fractions of *B. orientalis* was fractionated on normal phase HPLC, yielding a single behaviorally active 1 min fraction. Because of its behavior in flash column chromatography and co-elution with *B. germanica* keto-aldehydes, we suspected that this fraction would also contain aldehydes. The fraction was derivatized with DMH and analyzed by GC-MS in selected ion monitoring (SIM) mode (86 [aldehyde N,N-dimethylhadrazones McLafferty rearrangement], 464 [expected DMH product], 420 [M^+ -dimethylamide], and 393 [M^+ -(CH=\text{N}-\text{N(CH}_3)_2]); Fig. 5.2). The retention time of the peak with the appropriate relative abundance of the selected ion fragments was ~3 min earlier than that of DMH-derivatized 27-oxo-3,11-dimethylheptacosan-2-one. In addition, the active HPLC fraction was reduced using the modified Wolff-Kishner, and 11-methylheptacosane was detected by GC-MS. Combined, these data suggest that the active compound in the 10 and 20% ether fractions of *B. orientalis* is 27-oxo-11-methylheptacosan-2-one.
Fig. 5.1 Identification of the active component in the 2% ether fraction from flash column chromatography of *B. orientalis* cuticular extract: (a) GC trace of the 2% ether fraction (line; 30 female equivalents) and behavioral activity of 0.33 min fractions from preparative-GC (bars; n=30 males). None of the 30 *B. germanica* males responded to fractions where no bar is shown; (b) mass spectrum of the major peak found in the active fractions from preparative-GC; (c) mass spectrum of the active fractions from preparative GC after Wolff-Kishner reduction (see Methods).
Fig. 5.2 Selected ion monitoring (SIM) mode mass spectrum assigning 27-oxo-11-methylheptacosan-2-one to the courtship eliciting fraction. *B. orientalis* cuticular extract (10 female equivalents) was fractionated by flash column chromatography, the 10 and 20% ether fractions were found to be behaviorally active and were fractionated further by HPLC. The active HPLC fraction was derivatized with DMH and analyzed by SIM-MS.
**Discussion**

Our studies, like other recent investigations, have relied upon a behavioral assay wherein an isolated *B. germanica* male antenna is first extracted (hence, made neutral) and then augmented with behaviorally active fractions or compounds. The antennae of live *B. germanica* males are then manually stroked with this antenna to elicit the courtship response. Nishida and Fukami (1983) recognized, however, that male courtship is stimulated by a combination of chemosensory and tactile signals. Unlike most other contact pheromones which are active on any surface – for example, the Asian longhorned beetle courts plastic vials loaded with female contact pheromone (see Zhang et al. 2003) – extracts of sexually mature *B. germanica* females, or synthetic pheromone components, fail to release courtship when loaded onto artificial surfaces such as filaments or human hair. Remarkably, the pheromone also fails to elicit courtship when loaded on various insect antennae that differ substantially in fine morphological structure from those of the German cockroach (Nishida and Fukami 1983). All the evidence to date indicates, however, that specific courtship-eliciting compounds must be present to stimulate the behavior, and some insect antennae that do not normally elicit courtship in male *B. germanica* can be made to do so when augmented with pheromone. An interesting case, revealed in the present study, is that the antennae of *G. portentosa* females and nymphs elicit courtship in male *B. germanica*, but antennae of male *G. portentosa* do not. It will be interesting to learn whether this disparity is caused by sex- and stage- differences in cuticular chemicals or by the obvious sexual dimorphism in antennal morphology in this species. This species was also peculiar because extracts of behaviorally active whole insects were inactive. It is possible that highly abundant other cuticular lipids interfered with activity.

This study, to identify compounds that mediate interspecific courtship in male *B. germanica*, was motivated by two competing hypotheses: (a) that heterospecifics share one or more compounds with the contact sex pheromone of the female German cockroach; or (b) that the heterospecific compounds share common features with one or more *B. germanica* contact sex pheromone component, and therefore act as pheromone
analogs. First we showed that German cockroach males court five of 20 species of cockroaches in three families: Blattellidae (to which *B. germanica* belongs), Blaberidae, and the more distant Blattidae. These results complement the report by Nishida and Fukami (1983) that *B. germanica* males court the antennae of the cockroaches *Blattella nipponica* and *Blatta orientalis*, but not *Periplaneta fuliginosa* or *P. americana*. Interestingly, while we confirmed the lack of responses to the two *Periplaneta* species, we also showed that the closely related *P. australasiae* did elicit the courtship response.

We also confirmed earlier observations of courtship toward antennae of *B. orientalis* (Nishida and Fukami 1983), extended them to males, females, and nymphs of this species, and used extracts of this evolutionarily distant species from *B. germanica* to address our two hypotheses. The results compel us to reject the first hypothesis, and favor the second, that *Blatta orientalis* compounds act as *B. germanica* contact sex pheromone mimics because they share several common features that release the courtship response in males.

**Coarse-tuning of pheromone reception facilitates interspecific courtship in German cockroach males** In most sex pheromones, slight changes in pheromone structure, such as changes in chirality, or modifications in blend ratios, render the pheromone inactive or even behaviorally antagonistic or repellent (Baker et al. 1998; Mori 1998). The contact sex pheromone of the German cockroach, on the other hand, possesses several unusual features. First, each of six components that comprise the pheromone blend can independently elicit the full sexual response, albeit at different concentrations. Second, the most abundant pheromone components are not the most effective at eliciting courtship. Third, this contact sex pheromone includes apparently redundant features that can accommodate substantial structural modifications while retaining overall activity. For example, the C-2 carbonyl is indispensable for activity of 3,11-dimethylnonacosan-2-one, but its elimination only reduces, but does not eliminate, behavioral activity of 29-hydroxy-3,11-dimethylnonacosan-2-one (Nishida and Fukami 1983). However, reduction of the carbonyl to a hydroxy group significantly augments
activity of both methyl ketone and hydroxy-methyl ketone components. Nishida and Fukami (1983) similarly showed that adding bulkiness to either the C-2 or C-29 substituents decreased activity of pheromone mimics. Although a 3,11-methyl branching pattern is essential for activity, presence of only a 3-methyl or an 11-methyl only slightly reduces activity; behavioral activity is also diminished, but rarely eliminated, as the methyl groups are shifted away from these two preferred positions. And finally an alkyl chain of 29 carbons is most effective, but it can be lengthened or shortened with only a gradual, incremental loss of activity (Nishida and Fukami 1983).

Given this rather broad tuning of pheromone reception in *B. germanica* males, it is not surprising that 11-methylheptacosan-2-one and 27-oxo-11-methylheptacosan-2-one from *B. orientalis* can stimulate German cockroach males to engage in courtship behavior. Although behavioral activity of authentic compounds remains to be demonstrated, their unique structural features (27 carbon alkyl chain, C-2 carbonyl, C-11 methyl group, terminal aldehyde [formyl] group) are expected to impart them with behavioral activity. Based on the relative activity of various pheromone analogs, we predict that 11-methylheptacosan-2-one and 27-oxo-11-methylheptacosan-2-one will be less active than the respective native pheromone components 3,11-dimethylheptacosan-2-one and 27-oxo-3,11-dimethylheptacosan-2-one; moreover, the keto-aldehyde is expected to be slightly more active than its methyl ketone homolog.

**Features of German cockroach ecology that promote broad-spectrum tuning of male sexual response** Why, then, is the German cockroach so indiscriminate in his courtship behavior? First, we cannot reject the notion that the contact sex pheromone of female *B. germanica* might consist of many more structurally related, yet diverse components, including the C_{27} compounds identified from *B. orientalis*. This alone would naturally broaden the sexual response spectrum of males.

The female contact sex pheromone consists of three 29-carbon components (3,11-dimethylnonacosan-2-one, 29-hydroxy-3,11-dimethylnonacosan-2-one, and 29-oxo-3,11-dimethylnonacosan-2-one) and a homologous series of 27-carbon components.
The 29-carbon dimethyl ketone (and probably the 27-carbon homolog as well) is derived from insertion of a C-2 carbonyl into the preformed 3,11-dimethyl alkane (Chase et al. 1992), and a similar mechanism might give rise to the 29-hydroxy-, 29-oxo-, 27-hydroxy-, and 27-oxo- pheromone components. The epicuticular surface of *B. germanica* contains numerous methyl-branched alkanes in homologous series of 27- and 29-carbon chains (Augustynowicz et al. 1987; Carlson and Brenner 1988; Jurenka et al. 1989). It is possible that the same enzymes that catalyze the hydroxylation and oxidation reactions might recognize related hydrocarbons as substrates. Based on the structure-activity studies of Nishida and Fukami (1983), almost all the potential methyl ketone derivatives of the cuticular hydrocarbons—especially of 3-methyl- and 11-methyl-alkanes—would elicit courtship responses in males. If so, the broad tuning of the male courtship response may be a natural consequence of an unusually diverse contact sex pheromone blend in the female.

It is also plausible that a similar biochemical mechanism might operate in *B. orientalis*. The largest hydrocarbon peak identified in this species is 11- and 13-methylheptacosane (Lockey and Dularay 1986) and the 11-isomer could give rise to 11-dimethylheptacosan-2-one and then to 27-oxo-11-dimethylheptacosan-2-one. We did not detect the 27-hydroxy analog, but it is possible that it rapidly and highly efficiently converted to the keto-aldehyde. Nevertheless, these compounds do not appear to serve as sex pheromones in *B. orientalis*, as they occur in nymphs as well as adult males and females (data not shown).

There are many cases in which unrelated species—even from different classes (e.g., elephants and moths)—have converged on identical or similar chemicals for sexual communication. Given the relatively limited chemical moieties of long-chain cuticular lipids it is perhaps not surprising that unrelated insect species would independently evolve similar cuticular lipids to serve physiological or behavioral functions, or both. As long as species with overlapping sexual communication signals
remain separated by geography, space (microhabitat), or time, there is little imperative to diverge their shared sexual signals (more below).

In addition, we propose several major evolutionary forces that might have shaped qualitative (types of compounds, blends) and quantitative (blend ratios, response thresholds) elements of the sexual response of *B. germanica* males: (a) a highly male-biased operational sex ratio in this species; (b) obligate coupling of a specialized male urate excretion with copulation; (c) a shift toward reliance on a volatile pheromone for species-specific interactions; and (d) absence of closely related species in the anthropogenic environment leading to relaxation of selection on a finely tuned species-specific sexual communication.

Although the sex ratio in adult German cockroach populations is 1:1, the operational sex ratio in this mating system is highly male-biased. Females carry an egg case (ootheca) for the duration of the embryonic development, during which they are sexually unreceptive, and are only receptive for a short period of 2–3 days during each 25-day ovarian cycle. Moreover, females effectively store sperm and only a fraction of the adult female population mates more than once. Hence, the probability of mating is low for most males, and it is possible that they evolved low response thresholds to the pheromone components to efficiently detect females within very large aggregations.

Low behavioral thresholds and broad receptor tuning might also be favored and reinforced by a peculiar mechanism that *B. germanica* males have evolved to excrete nitrogenous waste. They sequester urates into specialized uricose glands, which can only be voided as a urate plug into the female during copulation. Protein-deficient females metabolize this nuptial gift and incorporate its constituent nitrogen into amino acids that they provision to embryos (Mullins et al. 1992). Adult males are therefore under strong selection to accumulate urates in their uricose glands, but males with urate-laden glands also are under strong pressure to mate because copulation is the only means to void potentially toxic urates. These opposing forces, together with low probability of mating, operate in the same direction to select for a low sexual response
threshold in males and broad response spectrum to various contact sex pheromone mimics.

Sexually receptive *B. germanica* females also emit a volatile pheromone, blattellaquinone (Liang and Schal 1993; Nojima et al. 2005) that could add species-specificity to the mate-finding process. This pheromone might diminish the need to fine-tune contact sexual signaling and signal reception. This idea is probably much under-appreciated in chemical ecology, as examples emerge of effective, sex pheromone-mediated species isolation in the field that may be circumvented at close range (in cages), resulting in interspecific mating (e.g., Groot et al. 2006).

Lastly, absence of closely related species in the anthropogenic environment might have relaxed selection for fine-tuned species-specific sexual communication in *B. germanica*. In sympatry, species uniqueness is selected upon and maintained through reproductive character displacement, resulting in divergent sexual signals and behavioral and morphological differences (Kaae et al. 1973; Wyatt 2005). Sexual interaction between closely related species is more commonly observed in species that have become sympatric due to recent introductions (Keenleyside 1967; Grant et al. 2002; Ardeh et al. 2004). The evolutionary history of cockroaches is poorly understood. Nevertheless, the fossil record and centers of extant species diversity suggest that while the genus *Blattella* probably originated in southeast Asia (Roth 1985), the five species that can elicit courtship in *B. germanica* probably originated in east and west Africa (Cornwell 1968). Whereas *Blatta orientalis*, *Supella longipalpa* and *Periplaneta australasiae* are commensal with humans and domestic animals (Cornwell 1968), the other two species, *Blaptica dubia* and *G. portentosa* are free-living and limited to tropical climates; the distribution of the latter (hissing cockroach) is limited to Madagascar. But even for the commensal species, interspecific encounters would be uncommon because they have different microhabitat preferences and mixed infestations of these species are rarely seen. Moreover, they are distant relatives and hybridization cannot occur between the German cockroach and any of the other species. Closely-related *Blattella* species, such as *B. nipponica* and *B. asahinai*, can release courtship
behavior in *B. germanica*, and the latter two can hybridize, forming fertile offspring. Even here, however, their spatial distributions do not overlap. It thus appears that male *B. germanica* might have broadened their sexual behavioral response spectrum under the artificial single-species “monoculture” environment in which they have lived for at least several thousand years.

It would be fascinating to know through comparative studies of congeners if broadening of the male antennal response spectrum has in turn resulted in novel female-produced pheromone components, and vice versa. The human-built ecological context might not only free *B. germanica* from interaction with closely related species and broaden its communication channel but it could also facilitate shifts in pheromone blend composition away from the ancestral blend and toward metabolically less costly blends.

Identification of the courtship eliciting compounds of other cockroaches might shed light on the range of compounds that the *B. germanica* pheromone receptor(s) will accept, and thus will enrich our understanding of the structure-activity relationship of pheromone reception in the German cockroach. It will also begin to address the question of whether these cockroaches share similar biochemical mechanisms that sequentially metabolize behaviorally inactive hydrocarbons to oxygenated active derivatives. Finally, these investigations should elucidate the functions that long-chain, cuticular methyl ketones, keto-alcohols and keto-aldehydes serve in various insects.

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