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Spider Pheromones – a Structural Perspective

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Abstract Spiders use pheromones for sexual communication, as do other animals such as insects. Nevertheless, knowledge about their chemical structure, function, and biosynthesis is only now being unraveled. Many studies have shown the existence of spider pheromones, but the responsible compounds have been elucidated in only a few cases. This review focuses on a structural approach because we need to know the involved chemistry if we are to understand fully the function of a pheromonal communication system. Pheromones from members of the spider families Pholcidae, Araneidae, Linyphiidae, Agenelidae, and Ctenidae are currently being identified and will be discussed in this review. Some of these compounds belong to compound classes not known from other arthropod pheromones, such as citric acid derivatives or acylated amino acids, whereas others originate from more common fatty acid metabolism. Their putative biosynthesis, their function, and the identification methods used will be discussed. Furthermore, other semiochemicals and the chemistry of apolar surface lipids that potentially might be used by spiders for communication are described briefly.

Keywords Amino acids · Citric acid · Lipids · Silk · Fatty acid biosynthesis

Introduction

Pheromone communication is an important information channel that is especially suited to transmit information and is used by many small organisms that live in a spacious environment, e.g., spiders. Although not obvious, spiders contribute by their carnivorous lifestyle in an important way to our environment, mostly by considerably reducing the numbers of other arthropods. Whereas the use of chemical information is not directly obvious in spiders, many studies have shown that they employ the chemical communication channel in various scenarios, such as mate finding, the initiation of courtship, or mate acceptance.

Compared with insects, the information obtained on the use of pheromones and the number of chemically identified pheromones of spiders is still low. Nevertheless, in recent years, evidence has shown clearly that pheromones and other semiochemicals are widely used by spiders from many different genera. The number of structurally identified spider pheromones remains small, especially compared with the number known from insects. This lack of knowledge can be explained not only by experimental difficulties during the identification process, e.g., problems of rearing large numbers of spiders, the small amounts of the produced pheromones, and problematic bioassay methods, but also by the lack of chemical expertise (see Schulz, 2004; Gaskett, 2007 for a detailed discussion). The lower direct economic impact of spiders compared with insects might also play a role. To date, more than 42,000 species of spiders have been described in over 100 families (Platnick, 2012), although the classification is still open to intensive debate. A recent reasonable phylogeny of spider families is given in Fig. 1 (Roth et al., 2005), which includes the few spider families for which information about the structure of their pheromones exists.

Several reviews have been published so far that illuminate the chemical communication of spiders from various perspectives (Schulz, 1997, 2004; Huber, 2005; Gaskett, 2007; Trabalon and Bagnères, 2010; Uhl and Elias, 2011), and the general natural product chemistry of spiders

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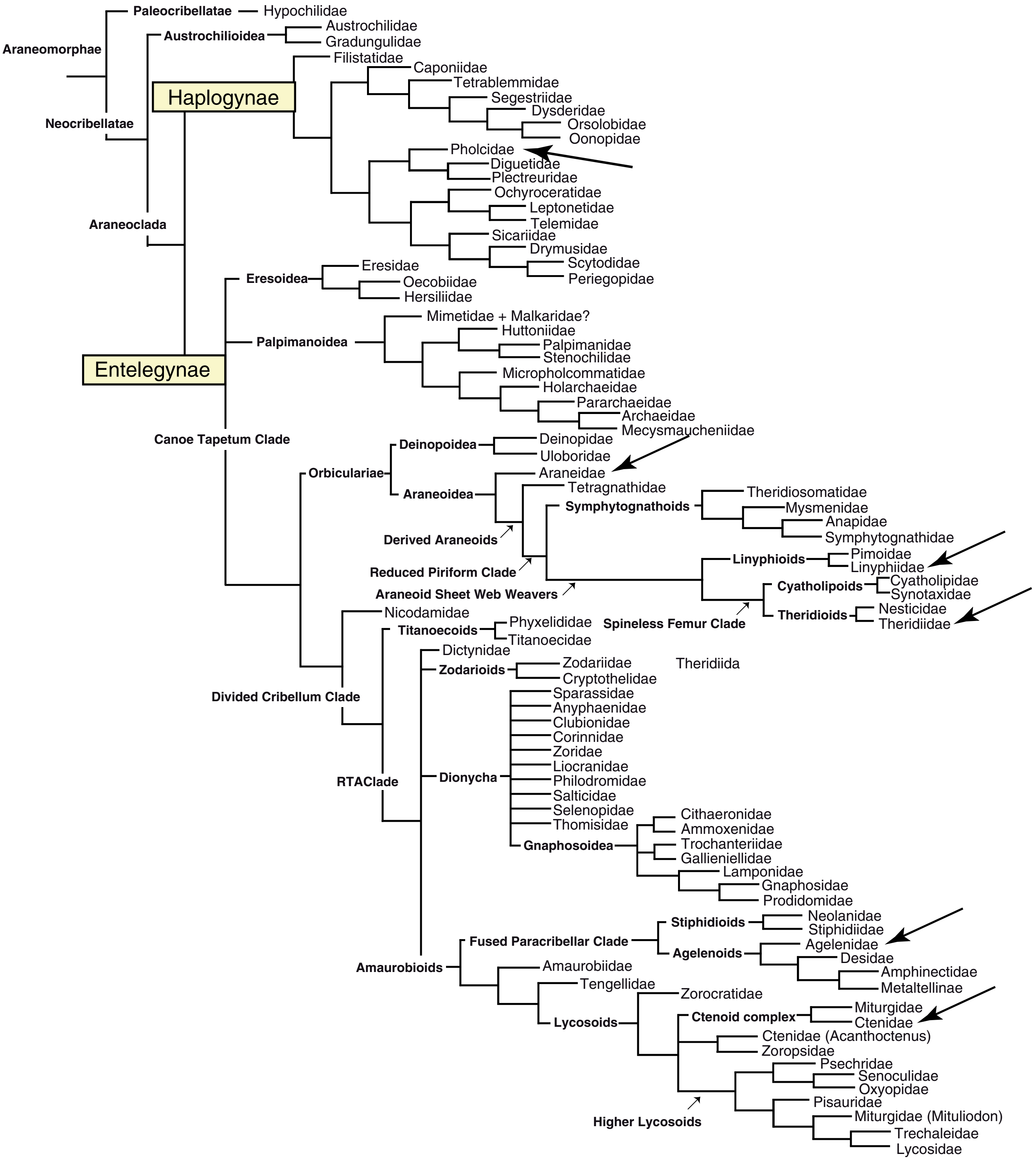

Fig. 1 Phylogeny of spider families according to Roth et al. (2005). Arrows indicate families in which pheromones have been identified

including venoms has been reviewed (Schulz, 1997; Olsen et al., 2011). In most of these review articles, the occurrence of pheromones in various spider families is highlighted.

No attempt is made here to duplicate this work, but rather a different structure-oriented perspective is taken, aimed at understanding pheromone usage in spiders and the function of their pheromone systems. Pheromones can be divided into various compound classes, depending on the biosynthetic pathways by which they are formed (Francke and Schulz, 2010). Lepidopteran pheromones, for example, originate from fatty acid metabolism and are typical secondary metabolites. The structurally identified spider pheromones will be discussed in this review in the context of their putative biosynthesis, although no direct study of pheromone biosynthesis in spiders has been published so far. Nevertheless, our knowledge of biosynthetic transformations obtained by natural product chemistry during the last few decades allows reasonable proposals to be made.

Each pheromone will be discussed in the context of the respective spider families, according to their phylogenetic occurrence (Fig. 1). This will reveal whether certain compounds or compound classes of pheromones used by spiders are mono- or polyphyletic. Furthermore, the behavior of the spiders in the pheromone context is reported. Hopefully, a better understanding of the function of the pheromone systems including their chemical properties will result.

Pholcidae

This family is the only haplogyne spider family in which pheromones have been characterized. They live in cellars, caves, and other undisturbed areas in which they build glueless webs; some of them resemble harvestmen. *Pholcus beijingensis* is a cave inhabitant in China. Webs of receptive females are attractive for males searching for mates. In a two-choice arena system, males chose dichloromethane extracts from receptive females over other types of silk extracts, including those of egg-holding, already mated females (Xiao et al., 2009). After individuals had been allowed to build their webs in small plastic boxes for 48 h, the silk was collected and extracted with dichloromethane; the extracts then were analyzed by GC/MS. The 13 identified compounds comprised aldehydes, ketones, esters, amides, and a hydrocarbon (Xiao et al., 2009), but included several possible anthropogenic contaminants, i.e., butylated hydroxytoluene from the plastic boxes in which the silk was collected, phthalates, and fatty acid amides, which are known lubricants. Octadecane also is unlikely to occur in spiders alone because of its even carbon number, whereas squalene might originate from the spider but is also a common contaminant from human skin.

Although no qualitative differences were detected between extracts of silk from receptive females, egg-holding females, or males, the abundance of (2*E*,6*E*)-farnesyl acetate (**1**) (Fig. 2), hexadecyl acetate (**2**), and the anthropogenic diisobutyl phthalate was markedly higher in the first case. Males then were tested in the two-choice arena system with synthetic compounds. A 2:1 combination of acetates **1** and **2** was highly attractive; none of the compounds was active alone (Xiao et al., 2009). Not surprisingly, the phthalate was inactive. 2-Heptadecanone (**3**), another possible pheromone candidate of slightly lower significance in the abundance analysis, was not tested.

Interestingly, silk from *Pholcus phalangoides* does not contain these compounds, but rather ketone **3** and its bishomolog 2-nonadecanone (unpublished results). (2*E*,6*E*)-Farnesyl acetate has been identified as a pheromone in click beetles (Yatsynin et al., 1996), and it is common in several secretions of bees and paper wasps. Hexadecyl acetate is a common constituent of many moth pheromone secretions, but its pheromonal activity has been established in only a few cases (van de Veire and Dirinck, 1986; Löfstedt et al., 1986).

The alkene (*Z*)-9-tricosene (**4**) has been identified as a male-produced signal that promotes courtship acceptance by females (Xiao et al., 2010). Whole spiders were immersed into dichloromethane for 24 h, and the extract was analyzed by GC/MS. (*Z*)-9-Tricosene was identified solely in the male extracts in an amount of about 0.1 μg per spider, whereas the ten other compounds identified, i.e., alkanes, aldehydes, palmitic acid, and the above-mentioned 2-heptadecanone, occurred also in extracts of receptive females and subadult males. Although dichloromethane is known to penetrate deeply into the skin, especially during the long extraction time used, all identified compounds were assumed to be present on the cuticle of the spiders.

Electrophysiology with tarsal chemoreceptors of females showed a strong response toward (*Z*)-9-tricosene, but only a weak one for the non-sex-specific compound tricosane. The time that the male required for courting the female was reduced by half in the presence of 1 μg of alkene **4**. The females remained motionless until the end of the courtship, when they finally approached the males and mating began. In these assays, the test compounds were placed on filter paper near the courting spiders in a test chamber (Xiao et al., 2010). In another assay, the attraction of females toward tricosene was not observed; the mating time was also not influenced (Xiao et al., 2010).

(*Z*)-9-Tricosene, that can now be regarded as the first chemically characterized signal of male spiders, is also found on the silk of female *P. phalangoides*; male silk was not investigated (unpublished results). It is a common

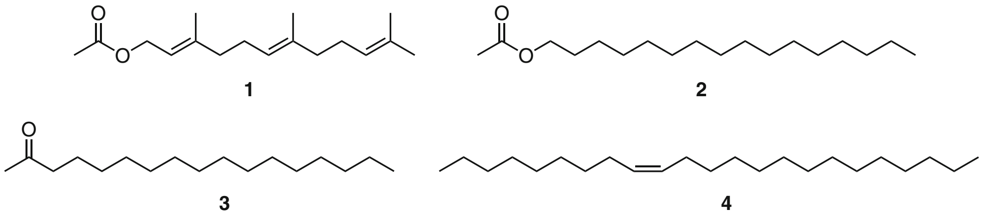

Fig. 2 Pheromones and other components from *Pholcus beijingensis*. (2*E*,6*E*)-Farnesyl acetate (**1**), hexadecyl acetate (**2**), 2-heptadecanone (**3**), and (*Z*)-9-tricosene (**4**)

constituent of many insect cuticles; furthermore, it is a pheromone of several insects such as the housefly *Musca domestica* (Carlson et al., 1971), a potential prey of *P. beijingensis* or the bee *Andrena nigroaenea* (Schiestl et al., 2000). The signal seems not to be highly specific and is active only in a given physiological or behavioral context.

The *Pholcus* pheromones hexadecyl acetate and (*Z*)-9-tricosene are biosynthetically derived from fatty acids. Their formation can be explained by common biosynthetic pathways known for many insect pheromones (Morgan, 2010). Stearic acid from the fatty acid pool is reduced to the respective alcohol and acetylated to form acetate **2**. (*Z*)-9-Tricosene (**4**) biosynthesis starts from oleic acid, another readily available fatty acid. Its elongation by three malonate units leads to the respective C_{24}-acid. Decarboxylation finally leads to tricosene (Morgan, 2010). The biosynthesis of farnesyl acetate **3** can be rationalized by the hydrolysis of the widespread terpene and steroid building block, farnesyl pyrophosphate, followed again by acetylation.

Araneidae

This entelegyne family is characterized by their formation of classical orb webs. Early studies showed that females of *Argiope trifasciata* can attract males of their own species and those of *A. aurantia* by a pheromone that does not seem to be species-specific (Enders, 1975). This phenomenon was more closely investigated in the European wasp spider, *Argiope bruennichi* (Chinta et al., 2010). The major volatile pheromone component was identified as trimethyl (2*R*,3*S*)-methylcitrate (**5**) by analysis of its mass spectrum, synthesis, and stereochemical analysis by enantioselective GC. It is accompanied by the minor (2*S*,3*S*)-enantiomer (**6**) in varying ratios between 25:1 and 6:1 (Fig. 3). These compounds can be identified in the headspace of living spiders or in extracts of their silk. The female spiders also produce this pheromone when confined to a small volume of about 200 ml, needed for collection of headspace volatiles. The exact location of the pheromone source is not clear; the pheromone can be delivered during silk production from the silk, but direct release from the body cannot be ruled out.

Although bioassays using a Y-tubed olfactometer have failed, a typical problem with spiders [see Gaskett (2007) for a detailed discussion of pheromone bioassays with spiders], field experiments with tripod traps on open meadows on sunny days have been highly successful in attracting spiders (Chinta et al., 2010). For example, with 10 traps loaded with 25 μg of a 6:1 mixture of the two citrates **5** and **6**, 34 males were caught in 30 min. Even 0.3 μg of the pheromone still showed activity. After arrival at the tripod, the males showed typical courtship behavior such as the application of silk strands from the trap to the vegetation, jerking on the substrate, and abdomen vibration upon contact with the source. These behaviors also are performed when a male arrives at a web of a female, thus generating vibrational cues that are transmitted by this web and received by the female. The relative proportion of **5** and **6** seems to be not highly critical, because both 6:1 and 2:1 mixtures have proved to be active. These experiments were the first successful applications of spider pheromones in the field (Chinta et al., 2010). Earlier attraction attempts in the field with the *Linyphia* pheromone discussed below failed. The production of the pheromone gradually rises after the final molt of the female until it reaches a peak on day 4. After day 8, only minor amounts are produced (S. Chinta, S. Schulz, unpublished results). Males and juvenile females do not contain the compounds. Adult virgin females of *A. argentata* and *A. blanda* also contain trimethyl methylcitrate, but stereochemical analysis or behavioral experiments have not been performed (S. Schulz, G. Uhl, S. Chinta, unpublished results). These data together with the observations of Enders (1975) suggest that trimethyl methylcitrate is broadly used by *Argiope* spiders as a sex pheromone. The webs of unmated adult *A. keyserlingi* also are highly attractive to males, but the structure of the pheromone has not been determined in this species (Gaskett et al., 2004).

Another adult female-specific compound of *A. bruennichi* is 3-octanoyloxy-4-butanolide (**7**). This unusual volatile might also play a role in the chemical communication system of *Argiope.* It occurs in virgin and mated females, but not in all individuals analyzed. A function as a sex pheromone is thus unlikely.

Pheromone **5** seems to be biosynthetically derived from the citric acid cycle of primary metabolism (Fig. 4). The enzyme citrate synthase usually forms citric acid (**10**) from oxalacetate (**9**) and acetyl-CoA. Under certain conditions, it can accept propionyl-CoA (**13**) instead of acetyl-CoA, forming methylcitric acid (**14**). This acid then can be esterified, probably by *S*-adenosylmethionine (SAM), to form the volatile methylcitrate **5**. Whereas in animals, an *si*-citrate synthase is active, forming the (2*R*,3*S*)- and (2*S*,3*S*)-isomers observed in *Argiope*, in microorganisms, a *re*-citrate synthase has been found, producing the opposite (2*S*,3*R*)- and

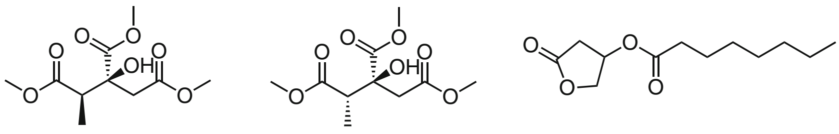

Fig. 3 Compounds discussed in the context of *Argiope* sex pheromones. Trimethyl (2*R*,3*S*)-methylcitrate (**5**), trimethyl (2*S*,3*S*)-methylcitrate (**6**), and 3-octanoyloxy-4-butanolide (**7**)

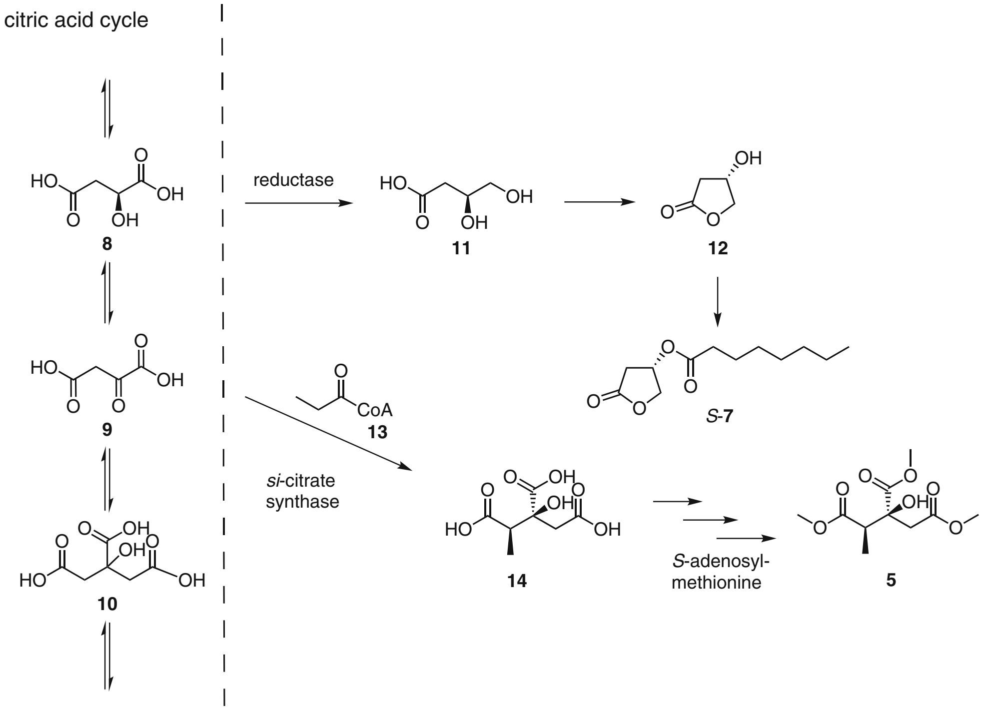

Fig. 4 Proposed biosynthetic pathway for trimethyl (2*R*,3*S*)-methylcitrate (**5**), supported by results obtained by Podebrad et al. (1999) for the biosynthesis of (2*R*,3*S*)-methylcitric acid (**14**)

(2*R*,3*R*)-isomers (Podebrad et al., 1999). The formation of the lactone **7** can be explained by the reduction of malic acid (**8**) of the citric acid cycle to form dihydroxybutanoic acid (**11**). After easy cyclization, acylation yields the lactone **7** in the *S*-configuration; nevertheless, the absolute configuration of **7** of *A. bruennichi* is unknown.

Linyphiidae

The first spider pheromones were identified in the extremely species-rich family Linyphiidae (Schulz and Toft, 1993b). Female linyphiids usually build horizontal sheet webs that are rich in silk. The estolide (condensation product of two hydroxy fatty acids) of two (*R*)-3-hydroxybutyric acid (**16**) units, (3*R*,3*R′*)-3-hydroxybutyryloxybutyric acid (**15**), is produced by adult virgin females of *Linyphia triangularis*, but not by mated or juvenile spiders. This compound is formed most likely during web production, and is present on the silk in large amounts of up to 5 μg/web (Schulz and Toft, 1993b). The compound has been identified by GC/MS, derivatization of samples, and stereochemical analysis by enantioselective GC. When males enter a female web, they start to cut threads and begin to roll up the silk into a small ball, presumably to reduce the evaporation rate of an attracting pheromone (Watson, 1986). This reduces the risk of an arrival of a competitor, because courtship can take several hours and can be interrupted by a male competitor. Only the silk of an adult virgin female induces this behavior, although it can also be evoked by estolide **15** when sprayed on other, previously inactive silk. Nevertheless, estolide **15** is not a highly stable compound and slowly degrades when exposed to the atmosphere, forming more volatile breakdown products, namely (*R*)-3-hydroxybutyric acid (**16**) and crotonic acid (**17**). The large amounts of estolide **15** serve in this way as a slow release formulation for the smaller compounds (Schulz and Toft, 1993b). Whereas (*R*)-3-hydroxybutyric acid (**16**) can evoke web-reduction behavior in males, the wrong enantiomer, i.e., (*S*)-3-hydroxybutyric acid or crotonic acid (**17**), are not able to do so. These results show that stereochemistry can play a role in the pheromone recognition of spiders, opposite to the finding in *Argiope*.

Several other linyphiids, exhibiting a similar mating behavior, also have been investigated for the presence of the estolide **15** on the silk of adult virgin females. The webs of unmated adult females of *Linyphia tenuipalpis*, which partially live in the same habitat as *L. triangularis*, contain similar amounts of **15** (Schulz and Toft, 1993b). The same is true for *Neriene peltata, N. emphana, N. litigosa*, and *Microlinyphia impigra*, whereas in *Neriene montana*, only traces of **15** are present (S. Schulz, S. Toft, unpublished results). The stereochemistry was determined to be (3*R*,3*R′*) in the investigated species *L.tenuipalpis* and *N. peltata*. The pheromone content during a summer season in Denmark was investigated in *L. tenuipalpis*. The pheromone amount rises in unmated adult females from the end of August from 1 μg per web to 5 μg until mid September. Then, it rapidly drops to about 0.1 μg, until it is absent at the beginning of October (S. Schulz, S. Toft, unpublished results). These data suggest that unmated females increase their effort to find mates until a deadline after which she is no longer likely to find a mate.

Behavior experiments have shown that males of *L. triangularis* and *L. tenuipalpis* exhibit the typical web-reduction behavior preferentially on webs of their own species, and react less often on webs of others, despite the use of the same web-reduction-inducing pheromone, (3*R*,3*R′*)-estolide **15** (Schulz and Toft, 1993b). Therefore, other species-discriminating cues must be present. Although physical differences in the silk might be possible, no data support this possibility. More likely, it seems to be the use of lipids that occur at high concentration on the surface of linyphiid silk (Schulz and Toft, 1993a). Whereas the different species mentioned above share common long-chain branched hydrocarbons on their silk, a large proportion of the surface lipids of the silk, up to 70 %, is made up by unique methyl-branched long-chain alkyl methyl ethers such as 1-methoxy-2,24-dimethyloctacosane (**38**, Fig. 9). In contrast to the hydrocarbons, they occur in species-specific mixtures, and might be used as species recognition signal by the males. The use of specific compounds should prevent false identification in the case of the use of common hydrocarbon-mixtures as signals, because contamination of a signature by prey hydrocarbons can thus be avoided. This idea has previously been explained in more detail (Schulz, 1997, 1999, 2004) but still awaits experimental verification. The *Pholcus* male pheromone (*Z*)-9-tricosene (**4**) and another fly pheromone, (*Z*)-9-octadecenyl acetate, also occur in the silk lipids of *Linyphia*, being especially prominent in juvenile stages (S. Schulz, unpublished results). Whether these compounds help in attracting flies as prey to the web needs to be explored.

Watson (1986) described, in his original paper, a bioassay for the testing of the volatile-mediated attraction of *L. litigosa*, thereby establishing the high attractivity of webs of virgin adult females. Y-shaped olfactometers usually cannot be used to test this attraction, because spiders tend to stay next to a wall and do not change this position. Therefore, Watson used a tube with an opening in the middle and two different cues at the two ends of the tube (T-shaped olfactometer). The residence time in the various arms then can be measured. This set-up also has been used with other linyphiids by S. Toft (unpublished results). Repeatable results were obtained only with *N. montana*. Whereas the estolide **15** showed weak attractivity, the monomer **16** was highly active, only being surpassed by the even more volatile crotonic acid (**17**). The webs of virgin adults were of similar attractivity as this acid. Again, the other enantiomer of **16**, (*S*)-3-hydroxybutyric acid, was inactive.

These results can be summarized into a coherent picture with regard to the biosynthesis and function of the *Linyphia* pheromone system (Fig. 5). (*R*)-3-Hydroxybutyric acid (**17**) is an intermediate of the fatty acid biosynthesis in all animals. It is formed in the first condensation step between an enzyme-bound acetyl unit and malonate coenzyme A. Two hydroxybutyric acid units or their enzyme-bound equivalents then can be condensed to the estolide **15**, which is deposited on web silk. On the silk, this acid slowly degrades forming, autocatalytically under the influence of moisture and air from the environment, the more volatile breakdown products 3-hydroxybutyric acid (**16**) and crotonic acid (**17**). This mechanism is supported by the presence of the trimeric compound **18** in low amounts on the silk, a compound that is also formed by an autocatalytic process. The spider thus has implemented an ingenious pheromone delivery system, ensuring a low but constant emission of volatile pheromone, while being able to perform other tasks, e.g., subduing prey or rebuilding web parts.

Interestingly, estolide **15** has been described as a bacterial degradation product of poly(3-hydroxybutyrate), the polymer of 3-hydroxybutyric acid, and a storage form of carbon of several bacteria (Reddy et al., 2003). Whether bacteria are involved in the production of the pheromone is unknown; however, if so, the spider would need a mechanism to selectively turn on and off the production of the pheromone by the bacteria, a seemingly unique mechanism.

Theridiidae

This large family of spiders builds three dimensional webs, and its members are often called cobweb spiders. An especially interesting genus is *Latrodectus*, also known as the widow spiders, including several species that contain venom that is toxic to humans. Some reports describe, in this genus, the presence of volatile and non-volatile cues that attract males and enable the discrimination of the female reproductive status (Ross and Smith, 1979; Anava and Lubin, 1993; Kasumovic and Andrade, 2004; Andrade and Kasumovic, 2005; Stoltz et al., 2007). Males of the Australian redback spider *L. hasselti* display courtship behavior with unmated adult females, but not with those of already mated females or juveniles. This behavior consists of increased movement, the addition of its own silk to the web, a ‘bouncing walk’ with flexing and extending legs, and exploratory movements (Stoltz et al., 2007).

The methanol extractable pheromone evoking this behavior was identified by GC/MS and NMR analysis of extracts of silk from unmated adult females, subadult females, and males. Synthesis and stereochemical analysis revealed the pheromone to be *N*-3-methylbutyryl-O-(*S*)-2-methylbutyryl-L-serine (**26**) (Jerhot et al., 2010). This compound is unique, because it defines a new class of natural products, the *N*,*O*-bisacylated serine esters. It also is a rare example of an amino-acid-derived pheromone in arthropods. In an arena bioassay, filter paper treated with 100 ng of the relevant compound triggered the courtship behavior of males (Jerhot et al., 2010). The stereochemistry is important, because only

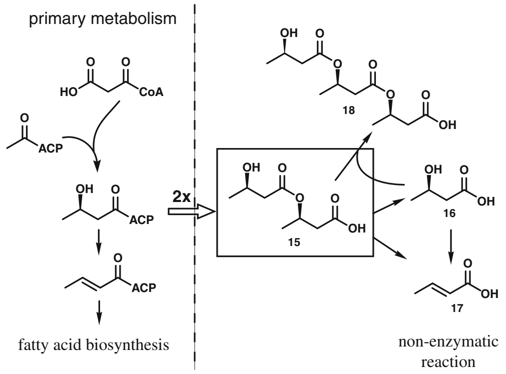

Fig. 5 Proposed biosynthesis and formation of pheromones of linyphiids. (3*R*,3*R*′)-3-Hydroxybutyryloxybutyric acid (**15**), (*R*)-3-hydroxybutyric acid (**16**), crotonic acid **17.** ACP: acyl carrier protein; CoA: coenzyme A

the correct *S*,L-enantiomer evoked the pheromonal response, whereas other stereoisomers were not attractive. A mixture containing compounds with the correct L-stereochemistry of the serine amino acid part, but racemic in the side chain stereochemistry, was not statistically different in effect compared with the control (Jerhot et al., 2010). Whether this indicates an inhibitory effect of the wrong stereoisomer or can be traced back to concentration remains to be elucidated. A related female-specific compound occurring in lower concentrations was *N*-3-methylbutyryl-O-methylpropanoylserine methyl ester (**27**) (Jerhot et al., 2010). *N*-Acetyl dopamine (**28**) was another female-specific silk component that proved inactive in the bioassay. Interestingly, the related *N*-acetyl tyramine (**29**) is present on the silk of unmated females of *Pholcus phalangoides*. Females of *L. hasselti* begin to add pheromone again, about three months after mating at the end of the mating season, in order to mate a second time (Perampaladas et al., 2008). In addition to the web-associated non-volatile pheromone, male *L. hesperus* spiders use unknown volatile chemical cues from female webs to locate them and to discriminate between different species (Kasumovic and Andrade, 2004).

The biosynthesis of **26** seems to be tightly related to the amino acid pool (Fig. 6). In addition to serine (**22**), the building blocks 3-methylbutyric acid (**21**) and (*S*)-2-methylbutyric acid (**25**) are needed. Both acids are usually formed from the amino acids leucine (**19**) and isoleucine (**23**), respectively, *via* transamination to the ketocarboxylic acids **20** and **25**, followed by decarboxylation. This is the common pathway for both acids and has been shown to operate also in arthropods (Adachi et al., 1985; Attygalle et al., 2007). Most likely, the methyl ester is formed by methylation with SAM (Nawrath et al., 2010), but the sequence of all these events leading to the serine derivative **26** is unknown.

Agenelidae

Females of the desert spider *Agenelopsis aperta* use a volatile pheromone to attract males and induce their complex courtship behavior (Riechert and Singer, 1995; Singer et al., 2000). The pheromone has been identified as 8-methyl-2-nonanone (**34**) by headspace GC/MS analysis of volatiles released by single individuals of adult, two-week-old virgin females, and juvenile females (Papke et al., 2001). The adult females released two compounds, namely the ketone **34** and 6-methyl-3-heptanone (**33**), that were absent in the juveniles. In a three-choice arena bioassay, male spiders were attracted to synthesized ketone **34**, but not to the other ketone **33**. The pheromone was attractive over several centimeters, even when only 0.5 ng was present on a filter paper in the arena. Courtship activity was also released by placing filter paper discs in the inactive webs of juvenile females. A dose as low as 1 pg ketone was able to release courtship including, among other behaviors, pedipalp drumming, abdomen waggling, and abdomen flexing in some males, whereas the effective dose (ED_{50}) was 27.5 μg (Papke et al., 2001). Ketone **33** showed no synergistic effect. *Agenelopsis aperta* males also use a unique quiescent pheromone that induces immobility in the female. It is released by the males during courtship, and has an effective distance of 3 cm. Its chemical nature is unknown (Becker et al., 2005).

The biosynthesis of 8-methyl-2-nonanone can be assumed to follow the pathway established for similar compounds in other organisms. The biosynthesis of the related ketone 9-methyl-3-decanone (**32**), only one carbon longer on the keto side than **34**, has been described in the bacterium *Myxococcus xanthus* (Dickschat et al., 2005). Leucine (**19**) again is converted to the acid **21** and activated by binding to an acyl-carrier protein (ACP). As is common in fatty acid biosynthesis, chain extension occurs by the addition of three malonate units **30**. The ACP-bound keto fatty acid **31** then loses the terminal carbon atom to form the ketone **34**. The longer chain in the bacterial ketone **32** arises by use of methylmalonate instead of malonate in the last elongation step (Fig. 7).

In late summer, male dust spiders *Tegenaria atrica* leave their webs in search of females. When arriving at the web of receptive females, the males start a vibrational courtship. This behavior can be elicited by extracts of silk from receptive females presented to the males (Trabalon et al., 1997). A pentane extract of the cuticle was prepared and separated by silica cartridges. The extract consisted of acids and their methyl esters and a few long-chain alcohols, ketones, and hydrocarbons (Prouvost et al., 1999). Only the fractions from female webs or cuticle containing acids or esters proved to be active. Additional bioassays showed the effectiveness of myristic, pentadecanoic, hexadecanoic, oleic acid, and linolenic acid and their respective methyl esters,

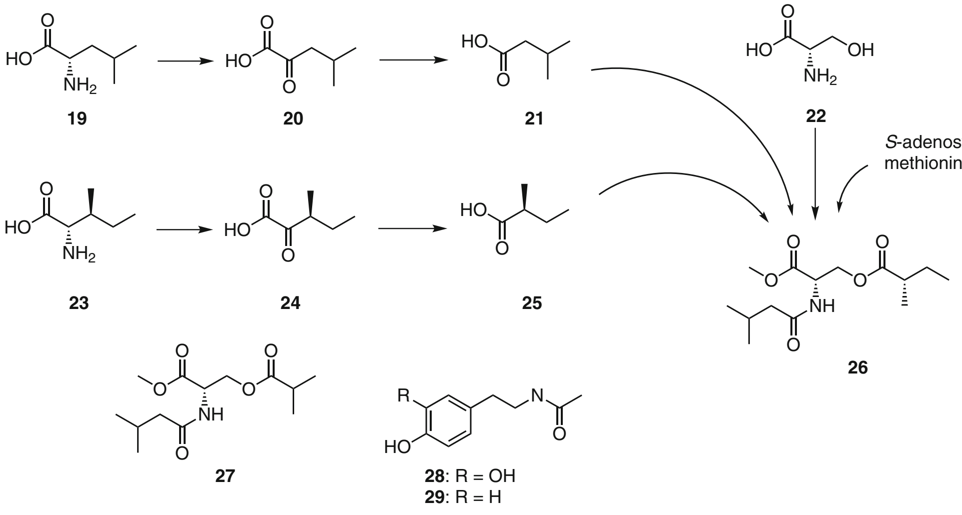

Fig. 6 Proposed biosynthesis of the pheromone *N*-3-methylbutyryl-O-(*S*)-2-methylbutyryl- L-serine (**26**) in *Latrodectus hasselti*. Other compounds discussed are *N*-3-methylbutyryl-O-methylpropanoylserine methyl ester (**27**), *N*-acetyl dopamine (**28**), and *N*-acetyl tyramine (**29**)

including methyl stearate, in eliciting the sexual behavior. This consisted of palpal drumming and prolonged physical contact with the pheromone-containing substrate. The bioassays were performed in an arena in which a filter paper impregnated with pure test compounds was presented (Trabalon et al., 2005). Each compound was tested at the concentration at which it occurs on the webs of receptive females. The duration of palpal drumming and physical contact with the filter paper was observed. The observed effects were roughly proportional to the amount of the compound tested (Trabalon et al., 2005). Trabalon et al. (2005) also found that the hormone 20-hydroxyecdysone regulates the amount of the compounds produced during web construction. Treatment with this hormone leads to increased levels of acids and methyl esters, whereas other silk constituents such as hydrocarbons or ketones remain unaffected. The attractive acids and esters also occur on the cuticle of female spiders in substantial amounts.

Each compound was tested at a different concentration, which makes comparison of their individual signal potency impossible. The results are however interesting because most of these acids are widespread in nature and can be found on the cuticle of many insects, and also on prey, although the concentration might vary widely, making these compounds relatively unreliable signal. The experiments also revealed that mixtures of compounds are of no importance here, because all active compounds were able to initiate courtship alone. Therefore, species specificity does not seem to play an important role in the courtship of this spider. These fatty acids are typical compounds of storage glycerides and are either synthesized by the spider or taken up during feeding and formed by the common fatty acid biosynthetic pathway. The methyl esters are probably formed by methylation with *S*-adenosylmethionine (Nawrath et al., 2010).

Ctenidae

The large hunting spiders of the family Ctenidae live mainly in warm areas around the globe, some of them being extremely poisonous to humans. The American wandering spider *Cupiennius salei* is a model organism for various biological questions (Prpic et al., 2008). The males wander through the environment when in search of females. When they come across single strands of the dragline silk of females, they probe it with their pedipalps. Adult females add a compound to the dragline silk that can be detected by the males. This compound evokes a typical vibrational courtship signal by the males, which wander through the environment to reach the usually less mobile female (Barth, 1993). The female responds, and thus the two sexes can

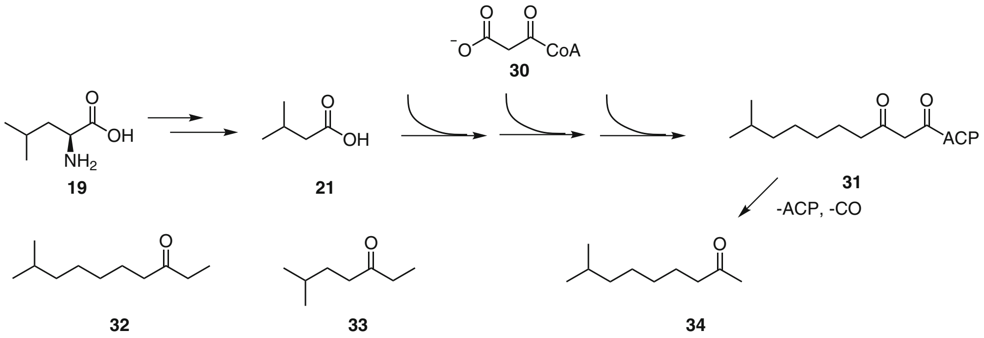

Fig. 7 Proposed biosynthesis of 8-methyl-2-nonanone (**34**), the sex pheromone of *Agenelopsis aperta*. Related ketones are 6-methyl-3-heptanone (**33**) and 9-methyl-3-decanone (**32**) for which a similar biosynthesis was proven in the myxobacterium *Myxococcus xanthus* (Dickschat et al., 2005)

locate each other. At close encounter, further courtship takes place, obviously without any chemical component. Thus, the female pheromone is only used as a courtship initiator, indicating the nearby presence of an adult female. The female-produced compound was identified by comparison of methanol extracts of active and inactive silk from juveniles or males by using NMR and turned out to be the unsymmetrical dimethyl ester of citric acid (**35**) (Papke et al., 2000). Interestingly, contrary to other spiders, the silk extracts did not contain any other small molecules detectable by NMR, indicating extremely clean silk. The absolute configuration was determined by synthesis and enantioselective GC to be a 95:5 *S*/*R*-mixture of the enantiomers (Papke et al., 2000). The biosynthetic formation of this pheromone, termed cupilure, most probably again starts from the citric acid cycle, followed by two stereoselective methylations, possibly by *S*-adenosylmethionine (Fig. 8).

The activity of the pheromone was tested with a cardboard tunnel. During the passage through the tunnel, male spiders encountered a single dragline strand of silk. Various conspecific silk types were tested. Because a relatively high proportion of male spiders react to any conspecific silk with vibration, every spider was tested twice, first with male silk, and second with male silk treated with **35**. About 40 % of the males reacted in both situations. Another 40 % did not react to male silk, but to treated silk. No male reacted first to male silk, but not to treated silk. The results were statistically evaluated by the McNemar test ($P<0.001$), which showed the high potency of the pheromone (Tichy et al., 2001). This type of bioassay was needed because of the inherent relatively high reactivity of males to any type of conspecific silk.

With the help of the synthetic pheromone, the first pheromone sensillum of spiders was identified. The sensilla are contact chemoreceptors that occur in large numbers on the dorsal surface of the pedipalps and contain 19 dendrites (Tichy et al., 2001). These tip pore sensilla are 0.2 μm in diameter. Electrophysiology has shown that cupilure (*S*-**35**) evokes high rates of response of the receptor cells, whereas the (*R*)-enantiomer and male silk is inactive (Papke et al., 2000; Tichy et al., 2001). Weak responses have been recorded with NaCl or saccharose. The amount of cupilure usually encountered during probing is calculated to be 180,000 molecules on a surface of 0.03 μm^2 (Papke et al., 2000; Tichy et al., 2001). The related species *C. getazi* and *C. coccineus* also contain **35**, although the absolute configuration has not been determined (S. Schulz, H. Tichy, unpublished results). Previous experiments have shown that *Cupiennius* males can discriminate between conspecific and heterospecific silk (Barth and Schmitt, 1991), although the NMR analysis of *C. salei* silk has revealed no other extractable components besides the pheromone as constituents. Therefore, the physical appearance of the silk probably also plays a role in species discrimination.

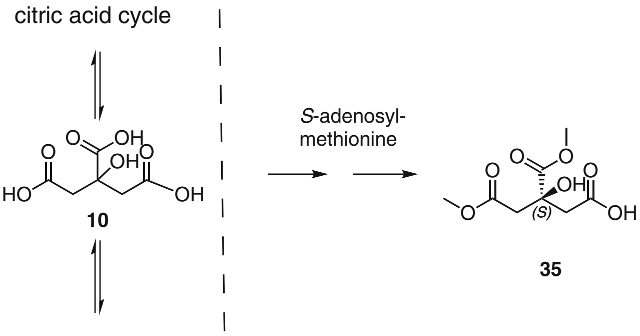

Fig. 8 Proposed biosynthesis of the *Cupiennius salei* pheromone cupilure (**35**)

Behavioral Evidence for Spider Pheromones

The above-discussed examples are the only ones in which the chemical structure of the spider pheromone has been clarified. Nevertheless, several other reports have been published showing the wide occurrence of pheromone use in spiders. The cases reported up until 2004 have been reviewed and listed in a table (Schulz, 2004). Since then, several additional reports have appeared. They are reported here, because many of these studies lay the groundwork for additional pheromone identification work that is needed for a better overview on the compounds that spiders use for communication.

The unique spider *Evarcha culicivora* (Salticidae) feeds indirectly on human blood by eating mosquitos after they have had a blood meal. Contrary to the behavior of most spiders, both sexes can initiate courtship when detecting chemical cues from the other (Cross and Jackson, 2012). Furthermore, olfactory signals also attract each sex to the other, independently of age or mating status. Only mated females show no interest in male spiders (Cross and Jackson, 2009).

The female sex pheromone of the wolf spider *Schizocosa malitiosa* (Lycosidae) has been isolated from silk but is not characterized (Baruffaldi et al., 2010). As in other *Rabidosa* sp., *R. santrita* males react to female dragline silk by dragline following and courtship (Brown, 2006). An additional study on another lycosid, *Schizocosa ocreata*, has shown that males can assess the reproductive status of a female by web-associated chemical cues (Roberts and Uetz, 2005). Furthermore, the signal seems not to be species-specific, because extracts of *S. ocreata* induce courtship in *S. rovneri* and *vice versa* (Roberts and Uetz, 2004a, b). This finding is consistent with the occurrence of identical pheromone components in several of the species discussed above. Males of *Pardosa milvina* use silk-bound chemical cues to discriminate between virgin and mated adult females (Rypstra et al., 2003). In the lycosid spiders *Allocosa brasiliensis* and *A. alticeps*, male volatile pheromones seem to occur that trigger female courtship in both species (Aisenberg et al., 2010). Nevertheless, in some species, chemical-mediated information transfer is unimportant for mate attraction or courtship, as in *Lycosa tarantula* (Fernández-Montraveta and Cuadrado, 2009).

Some spiders such as the crab spider *Misumena vatia* (Thomisidae) can distinguish between silk of different physiological states and different sexes based on the physical properties of the silk and most likely not by the perception of pheromones (Anderson and Morse, 2001; Leonard and Morse, 2006). Sociality exists to some extent in the comb-footed spider *Anelosimus studiosus* (Theridiidae). Female webs attract males, but they prefer the social phenotype over the solitary one (Pruitt and Riechert, 2009). In an unidentified spitting spider of the genus *Scytodes* (Scytodidae), females were found to make an odor-based mate choice that resulted in higher egg numbers, among other changes, compared with matings with less-preferred males (Koh et al., 2009). The tarantula *Brachypelma vagans* (Theraphosidae) chooses burrows inhabited earlier by conspecifics over new burrows. Most attractive are hexane extracts of female silk (Dor et al., 2008).

Other Semiochemicals

Semiochemical perception also occurs in encounters other than mating. A short overview will be given in order to assess under which circumstances semiochemicals are used by spiders.

Bolas spiders use kairomones to lure prey. They produce prey sex pheromones that attract moths and flies (Haynes et al., 1996, 2002; Gemeno et al., 2000; Zhu and Haynes, 2004). The attractive compounds have been identified in several cases. Chemical cues emanating from predatory spiders warn prey of a potential danger (see, e.g., Persons et al., 2001). *Latrodectus hesperus*, in contrast, can detect chemical cues from its prey to select a suitable habitat (Johnson et al., 2011). *Nephila edulis* places decaying matter in its webs to attract flies as prey (Bjorkman-Chiswell et al., 2004), a strategy for catch-rate enhancement. Parasitic spiders remain undetected in ant colonies in which they live on the ants resources by using chemical mimicry (Allan et al., 2002; Elgar and Allan, 2004, 2006; Witte et al., 2009; von Beeren et al., 2011). The hydrocarbons are acquired either by physical contact or through preying on the ants, and have been identifed. Ant pheromones themselves function as allelochemicals for ant-hunting zodariid spiders to locate their prey (Allan et al., 1996; Cárdenas et al., 2012). The responsible compounds have been identified. *Paratrechalea ornata* (Trechaleidae) males use nuptial gifts, the acceptance of the female being triggered by chemical cues from the gift (Brum et al., 2012). These aspects have been discussed elsewhere in more detail (Schulz, 2004; Uhl and Elias, 2011).

Silk and Cuticular Lipids as Potential Chemical Signals

Not only the cuticle, but also the silk of many spiders is covered by non-polar lipids. In insects, such compounds, predominately hydrocarbons, are well known to serve information transfer in many cases (Blomquist and Bagnères, 2010). This seems also to be true for spiders, because several examples have shown the importance of surface-lipid-associated cues. Their chemistry has been reviewed previously (Schulz, 1997, 1999; Trabalon and Bagnères, 2010). As in insects, hydrocarbons are a major surface lipid class. Nevertheless, spiders seem to produce a wider variety in compound diversity.

The American social spider *Anelosimus eximus* contains propyl esters of methyl-branched long-chain fatty acids, such as propyl 4,20- and 4,30- or 6,20- and 6,30-dimethylhentriacontanoate, as major components of its cuticle (Fig. 9) (Bagnères et al., 1997). The last quarter of the lipids is made up of fatty acids, their methyl esters, and linear and methyl-branched hydrocarbons (Bagnères et al., 1997). Long-chain methyl-branched 1-methoxyalkanes, such as **38**, are the dominating compounds of the lipids of the silk of *Linyphia triangularis* and other linyphiids (Schulz and Toft, 1993a). Their potential for signal transfer has been discussed above. This ether-specific species signature is potentially useful for species recognition and might have evolved to be a reliable signal, not being disturbed by prey hydrocarbons. In insects, these ethers have been found only in small amounts in *Pieris* butterflies (S. Schulz, unpublished results).

The most comprehensive investigation of silk lipids has been performed on *Nephila clavipes* silk (Schulz, 2001). Again, 1-methoxyalkanes (e.g., **37** and **38**) are the dominant compounds, accompanied by hydrocarbons with up to four methyl branches, alkanols, 1,3-alkanediols (e. g., **39**), and glyceryl ethers, fatty acids, and their respective methyl esters. Interestingly, even-numbered 2-methylalkanes (**41**) are the most prominent group of alkanes present. These results, together with the behavioral data reviewed above, show the high potential of surface lipids as chemosignals in spiders.

In addition to the work on *Tegenaria* presented above, other studies have shown the involvement of cuticular compounds in the chemical communication channel of spiders. In the subsocial spider *Stegodyphus lineatus* (Eresidae), kin recognition of spiderlings might be mediated by cuticular hydrocarbons (Grinsted et al., 2011), as has been shown by bioassay with cuticular extracts. Furthermore, their hydrocarbon profiles change during development. These mixtures consist of 38 compounds made up from linear alkanes and mono- and dimethyl-branched alkanes. The latter seem to be the relevant cues. The authors propose these to be less diet-dependent than are unbranched hydrocarbons and postulate a genetic dependence of their patterns.

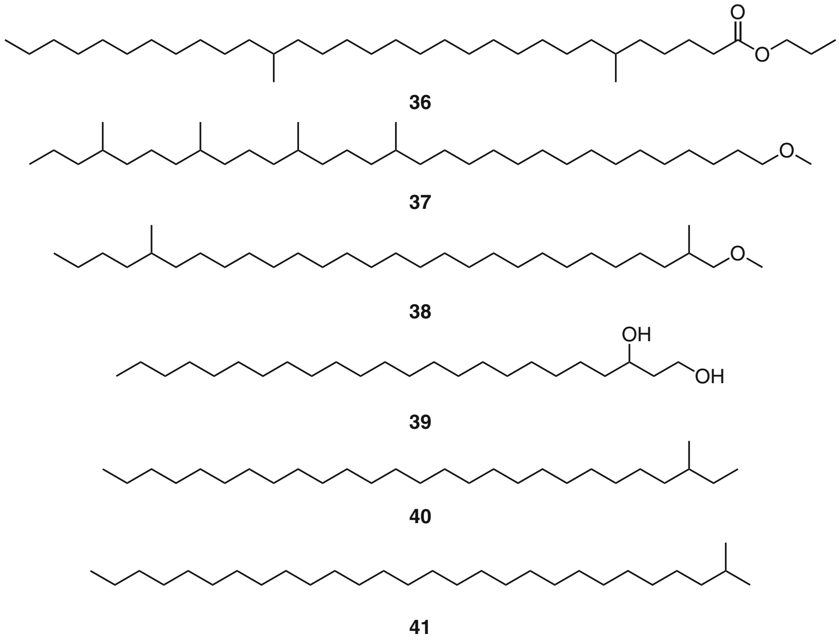

Fig. 9 Representative examples of compound classes occurring on bodies or silk of spiders. Propyl esters such as propyl 6,20-dimethylhentriacontanoate (**36**) in *Anolesimus eximus*, 1-methoxyalkanes such as 1-methoxy-16,20,24,28-tetramethylhentriacontane (**37**) or 1-methoxy-2,24-dimethyloctacosane (**38**) in *Nephila clavipes.* Ether **38** also occurs in *Linyphia triangularis.* 1,3-Tetracosanediol (**39**) is an example of diols from *N. clavipes*, which also contains 2-methylalkanes such as 2-methylocatcosane (**41**). The lipid layer of *Coelotes terrestris* contains branched alkanes such as 3-methylheptacosane (**40**)

In another subsocial spider, *Coelotes terrestris* (Amaurobiidae), spiderlings and females can discriminate webs and extracts by physiological status. Whereas adult females prefer webs of their own status, gregarious young spiders behave differently and prefer parental female silk (Trabalon and Assi-Bessekon, 2008). Cuticular lipid correlation analysis has revealed characteristic changes in their profiles, made up from unbranched and branched alkanes with up to two methyl groups, long-chain alcohols, and fatty acids. Whether these differences are really responsible for the observed behavior remains unclear. The lipid layer consists of 48 compounds including heptacosane, 3-methylheptacosane (**40**), palmitic acid, 1-docosanol, and 1-hexacosanol (Trabalon and Assi-Bessekon, 2008).

The lipids of *Brachypelma albopilosa* (Theraphosidae) including their cuticle components have been analyzed and evaluated in the context of agonistic interactions (Trabalon, 2011). These lipids are made up by mostly methyl esters of common C_{16}- and C_{18}-fatty acids, accompanied by free acids and linear alkanes between C_{13} and C_{34}.

Conclusion

During the last few years, we have obtained a better picture of the pheromones used by spiders. The compounds identified seem to be different from those used by insects in some cases (*Linyphia, Cupiennius, Latrodectus*, and *Argiope*) and are biosynthetically more closely related to the basic metabolic processes of living organisms. Some, such as trimethyl methylcitrate **5**, serine ester **26**, and cupilure **35**, are not known from other natural sources. In other cases, e.g., *Agenelopsis* and *Pholcus*, a biochemical similarity to insect pheromones is obvious. To gain a better overview of the chemistry, more structural identifications have to follow the numerous studies showing the existence of pheromones in many species. This will allow us to analyze the physicochemical properties of the pheromones and their relationship to their function. Such an analysis, as exemplified in the discussion of estolide **15** in *Linyphia*, can be important if we are to understand the actual function and evolution of these signaling systems. The investigation of pheromone biosynthesis should lead to the detection of the enzymes responsible for their production, and genetic analysis should lead to an understanding of their regulation.

When looking at spider phylogeny (Fig. 1) and the compounds identified to date, no clear trends for specific biosynthetic products used for spider pheromones are visible. Nevertheless, citric acid derivatives are present in two unrelated Ctenidae and Araneidae. The relatively closely related Linyphiidae and Theridiidae do not share common or related compounds, but at least rely on the fatty acid biosynthetic pathway or the respective fatty acid pool. The pheromones of pholcids or the Agenelidae are similar to insect pheromones.

Another pattern becoming evident is the lack of species specificity of many pheromones. Other differentiation methods such as habitat separation and visual or tactile cues are also important, diminishing the need for a species-specific pheromone in the first place. Furthermore, the function of cuticular and silk lipids seems to be not well understood. Apart from information transfer, these lipids also play a role in protection of the cuticle and the silk against water loss or against degradation by, for example, oxidation or attack by microorganisms, and might be important for the proper function of the silk material. Therefore, the lipid compounds have to fulfill several roles.

Further work is necessary on aspects of pheromone production, such as location, enzymes, and regulation and on sensory aspects in order to locate receptors and explore signal transmission and processing. Compared with insects, only a tiny fraction of the spider pheromone world has been explored. Nevertheless, their large predatory influence on the insect population and their ecological impact make it highly worthwhile to delve deeper into the pheromones of spiders.

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