



An airborne female pheromone associated with male attraction and courtship in a desert spider

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Spiders are exemplary loners: most species are solitary, territorial, and even cannibalistic throughout much of their life spans. Communication between individual spiders involves agonistic signalling in the form of vibratory and visual displays, which serves to maintain a widely dispersed population. Pheromones, however, have been implicated in bringing the sexes together during reproduction. We identified two potential sex pheromones collected from virgin, sexually mature females of the desert spider, *Agelenopsis aperta*, and used the synthetic compounds to test conclusions reached from previous behavioural trials indicating that chemical cues emitted by this female class attract courting males. We also investigated the role of chemical communication in prescribing the complex male courtship sequence. In separate trials, one of the synthesized pheromones, 8-methyl-2-nonanone, was found both to attract males to the source of the cue and to elicit courtship behaviour from them at very low dosages. Pheromonal cues release most elements and stages of the male courtship sequence in *A. aperta*. Two fundamental differences were observed, however, between the courtship males displayed in the presence of pheromone alone versus in the presence of a sexually receptive female. (1) Behaviour patterns associated with locating a female were significantly more frequent in the pheromone-alone treatment sequences. (2) Male actions, such as wave legs, lunge and retreat, were observed only in the female-present trials. These latter acts were displayed in response to female actions made towards the courting male.

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While pheromones are generally considered the primary mechanism of sexual attraction in most animal systems, simple chemical signals have been replaced by complex blends of pheromones (e.g. in insects) and by other sensory modalities (e.g. visual displays in birds) as selection has favoured messages that more effectively reach receivers, have greater specificity, and convey more information. Thus, from the study of insect pheromone systems, we have found that while single pheromones may attract males to females or release courtship behaviour, blends of pheromones augment the aggregative response and are required, often in combination with other non-pheromonal stimuli, for completion of the courtship sequence (Payne et al. 1986).

Spiders display both complex vibratory and visual signals in their interactions over webs, mates and territories (see review in Riechert 1982). Perhaps the ease of

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detection and measurement of these signals explains the fact that chemical communication in the group has been considered less important than other forms of communication. Emphasis on vibratory and visual communication systems has resulted in a general lack of knowledge of spider chemistry compared with other arthropod groups (Schulz 1997). The only pheromone identified from a spider to date is a contact pheromone that elicits web-reduction behaviour in males of the sheet-line weaver, *Linyphia triangularis* (Linyphiidae) (Schulz & Toft 1993).

The desert spider, *Agelenopsis aperta* (Gertsch) (Araneae, Agelenidae) has a complex courtship sequence involving several stages and numerous vibratory and visual displays and actions (males display 21 different action patterns; females, 16) (Singer et al. 2000). Nevertheless, recently completed behavioural studies indicate that male *A. aperta* respond with courtship to a volatile pheromone emitted by sexually mature virgin females. In this paper we investigate the pheromonal system associated with male spider attraction and courtship in *A. aperta*. We use synthesized pheromone to test the validity of the results presented in Riechert & Singer (1995), as well as to

investigate the role of chemical communication in pre-scripting the complex courtship sequence in this spider, *A. aperta*.

METHODS

Chemical Procedures

Pheromone identification

We screened the following classes of *A. aperta* for potential pheromone emissions: adult males, juvenile females, recently matured virgin females, virgin females that were 2 weeks past their final moult, and adult females that had mated. In contrast to many arthropods, the pheromone-producing glands in spiders are not known. We thus applied two screening techniques to collect emitted volatiles: abdomen wipe and headspace extract. We collected the abdomen wipe samples from restrained spiders, using the restraining device first described in Riechert (1984), a 23 cm³ syringe, to which we made the following two modifications: (1) we removed (sawed off) the needle insert end; (2) we levelled the rubber cap of the plunger. We used the plunger to position a spider introduced into the syringe against a cotton net covering placed over the open end. We rubbed the dorsum of the abdomen of the restrained spider with a small piece of filter paper. We extracted compounds from the filter paper with pentane or dichloromethane.

We used a modified closed loop stripping apparatus (CLSA) (Boland et al. 1984) in collecting the headspace samples. This technique is particularly suited to the collection of low amounts of volatiles in combination with minimized contamination from the environment. Briefly, we placed single individuals in a round-bottom glass flask (50 ml), which was connected to a small glass tube holding a filter equipped with 1 mg active charcoal. A membrane pump sucked the air through this arrangement and circled it back to the spider. After 8 h, we extracted the filter three times with 10 µl freshly distilled carbon disulfide and combined the solutions for further analyses. This procedure enabled analyses of volatiles emitted from single individuals.

We analysed both sets of samples by gas chromatography–mass spectrometry (GC–MS). Compounds were identified unambiguously by their mass spectra, their gas chromatographic retention times and comparison with synthetic samples. Gas chromatographic and GC–MS investigations were performed using a 30 m BPX-5 fused silica column (SGE) and a Mass Selective Detector (Hewlett Packard).

Syntheses of identified potential pheromones

The ketone 8-methyl-2-nonanone. The commercially available alcohol 5-hexen-1-ol was transformed into its tosylate by known procedures and subjected to a couïer catalysed coupling with 2-methylpropyl magnesium bromide (Fouquet & Schlosser 1974). The resulting 8-methyl-1-nonene was oxidized by a Wacker procedure using palladium chloride (Tsuji 1984) to yield the desired ketone, which was purified by column chromatography (purity >99%).

The ketone 6-methyl-3-heptanone. This compound was synthesized by reaction of propionyl chloride with 3-methylbutyl magnesium bromide at –78°C according to a standard procedure (Tietze & Eicher 1991).

Behavioural Analyses

Tests for biological activity of pheromones

We tested the effectiveness of both synthetic ketones in attracting male *A. aperta* and in evoking courtship. To obtain different concentrations of the test compounds, we diluted them in pentane. An initial concentration of 100 mg of pheromone/1 ml solvent was used in testing the biological activity of the pheromones. The concentration was diluted one order of magnitude in successive trials. In all of the experiments, 50 µl of the given solution was applied by pipette to a 2.1-cm circular disc of glass microfibre filter paper.

Release of male courtship. We determined whether each compound would elicit male courtship in trials involving empty webs, previously produced by juvenile spiders. We tested 10 adult male *A. aperta* in each control (solvent only) and treatment set of trials. We tested each male previously for sexual maturity by placing it on a web with a receptive female and checking for courtship behaviour (i.e. pedipalp drumming, abdomen waggle, flexing; see Table 1 for definitions). We removed the male before mating was initiated and allowed 1 week to elapse before testing the male's reponse in the volatile compound trial. Each male was used in only one trial of one test. A minimum of 10 males were tested in each trial.

At the initiation of each courtship trial, we placed the filter-paper disc to which we had either applied a particular concentration of a ketone in solvent or a disc to which solely the solvent had been applied (control) on the empty juvenile web. We placed the disc at the centre of the sheet-web and then introduced the male test subject. We observed each web for 2 h to detect courtship signalling. We used the stepwise dilution series of the synthetic components described earlier in different trials to determine the dose–response curve for each potential pheromone.

We also completed one trial involving a blend of the two ketones collected from sexually mature female *A. aperta*. The particular blend used was dictated by the results of the trials with the respective chemicals: 1×10^{-9} mg of nonanone: 1×10^{-1} mg heptanone.

Attraction of males. We completed the initial investigation of a potential attraction effect of the two pheromones collected from *A. aperta* in an arena choice system (Fig. 1). This arena system has been used in previous behavioural assays of male response to female chemical cues in *A. aperta* (Riechert & Singer 1995). We released each test male into a central arena (circular plastic container of 15 cm diameter and 6.5 cm height) permitting access through short tunnels (2.5 cm in diameter and length) into three peripheral (treatment) arenas of the same dimensions as the release arena. We recorded the

Table 1. Description of behaviour patterns displayed in *Agelenopsis aperta* courtship sequences

Behaviour pattern	Description
Level 1	Detection/location
Move	Undirected walk on web
Adjust position	Picking up legs and shuffling body within same location
Search	Short jerky movements on web with frequent direction shifts
Bite or pluck web	Short pull on silk threads with chelicerae (bite) or front legs (pluck)
Level 2	Signalling
Abdomen waggle	Swinging abdomen to left and right while moving across web. Often accompanied by silk laying
Drum	Rapid alternate placement and lifting of accessory front legs (pedipalps) on and off web
Palpate	Exploration of web with first pair of walking legs
Flex	Push on web with walking legs while raising body, followed by leg retraction with body lowering
Level 3	Making contact
Orient	Turn towards disc or female spider and stop often, with front legs spread apart forming an obtuse angle
Approach	Walk towards disc or female spider
Retreat	Runs in opposite direction of female spider
Jump	Short motion in which all eight walking legs leave web surface with spider landing at same location
Attack	Lunge towards female spider
Wave legs	Slow alternate raising and lowering of front legs from initial coplanar orientation above the web
Level 4	Self-preparation
Vibrate abdomen	High-frequency shaking of body (tremor), observed at end of some web-flexing events
Groom/preen	Rubbing pedipalps with first or second pair of legs on one side of the body at a time or rubbing walking legs against one another or on the side of the thorax or abdomen

Levels refer to temporal stages within the courtship sequence from transition analyses presented in Singer et al. (2000).

movement and behaviour of each male over a 2-h observation period within the arena system. Males displaying escape responses upon release were removed and retried at a later date.

In this attraction experiment, we positioned a filter paper disc at the far edge of each of the three treatment arenas. Two of the treatments had 50 µl of chemicals applied to the paper discs: solvent alone in one case and a synthetic compound dissolved in solvent in the other. No webs were present in any arena and each system was washed with 70% ethanol between trials. The placement of treatments in the arena system was randomized with

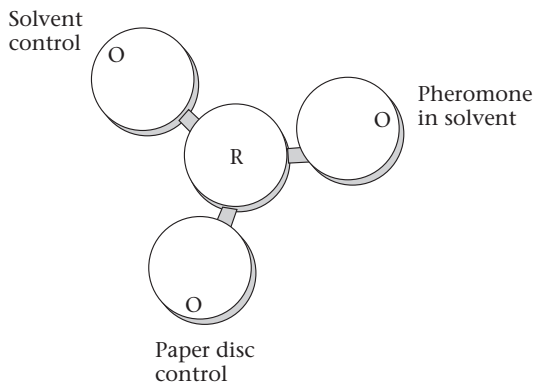


Figure 1. Three-choice arena system used in assessment of male *A. aperta* spider attraction to a potential pheromone. Male spiders were released into the central release arena (R) following establishment of different treatments in surrounding choice containers. All three-choice arenas had a central filter-paper disc. Two drops (50 µl) of the solvent pentane were administered to one disc, and the same quantity of a given concentration of pheromone in pentane was administered to a second disc.

each trial. All males were tested only once in this experiment and a minimum of 10 trials were completed in each set. We used a high concentration (1×10^{-1} mg of pheromone/1 ml of solvent) in the initial trials. We completed another trial offering a low pheromone concentration in the case of the compound showing significant levels of male attraction.

Influence on courtship sequence. In addition to female emission of a pheromone, the behaviour a male *A. aperta* displays during courtship may be influenced by such factors as: (1) the female’s load on her web (body mass); (2) female movements, which might provide vibratory or visual signals; (3) tactile cues. We compared the courtship sequences males displayed in the presence of sexually mature female *A. aperta* to those they displayed to the synthetic pheromone, the ketone 8-methyl-2-nonanone, which was presented on webs built by juvenile spiders that were removed before completion of the trial. The two treatments were: (1) a virgin female that was at least 2 weeks past the final moult, and (2) 50 µl of 1×10^{-2} mg of pheromone (8-methyl-2-nonanone)/ml solvent on a filter paper disc placed in the centre of a web produced by a juvenile *A. aperta*. This ketone was identified from trials completed earlier in the study as a male attraction and courtship pheromone in *A. aperta*. The juvenile spider was removed before setting up the treatment. We completed 16 trials for each treatment, with each male, female and web used in only one trial. We recorded all behaviour displayed within 15 min of the first action that typified courtship (i.e. pedipalp drumming, abdomen waggle, web flexing; Singer et al. 2000) in the order in which it occurred using an open-format event recording system (Noldus Observer).

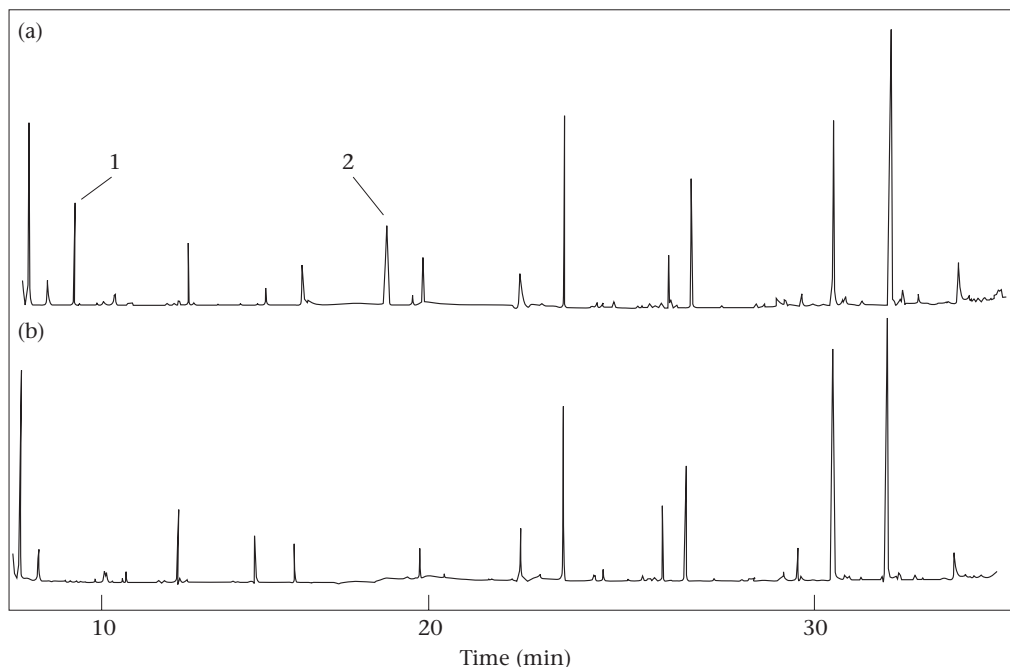


Figure 2. Total ion chromatograms of closed loop stripping apparatus (CLSA) extracts from a single virgin *A. aperta* female that was 2 weeks past her final moult (a) and a juvenile *A. aperta* female (b). 1=6-methyl-3-heptanone; 2=8-methyl-2-nonanone.

RESULTS

Identification of Potential Pheromones

About 20 different compounds regularly were identified in the CLSA samples, albeit always in low quantities. These components were mostly unbranched hydrocarbons with chains of varying length, C_{10} – C_{30} , accompanied by a smaller amount of branched or unsaturated hydrocarbons. Oxygenated compounds like short chain aldehydes (e.g. nonanal) also occurred. Some of these compounds are most likely to be contaminants not originating from the spider. They were present in all samples and therefore not considered as potential pheromones.

Two compounds were present only in samples obtained from virgin females that were 2 weeks past their final moult (Fig. 2). The early eluting compound could be readily identified as 6-methyl-3-heptanone by its mass spectrum and comparison with an authentic sample. The later eluting compound exhibited a mass spectrum with strong ions at $m/z=43$ and 58, typical for 2-ketones, and a molecular ion at $m/z=156$ (Fig. 3). It resembled the spectrum of 2-decanone, but the gas chromatographic retention time on an apolar phase was shorter than that for 2-decanone, pointing to a branched 2-ketone. Iso-branched aliphatic compounds very often exhibit mass spectra similar to respective unbranched compounds. Therefore we concluded that the unknown compound could be 8-methyl-2-nonanone. After synthesis of this compound and comparison of mass spectra and retention times with those of the natural compound, this proved to be correct.

The analyses of the abdomen wipes revealed the presence of a large number of lipidic compounds. (In the

broad sense, the two methylated ketones, 8-methyl-2-nonanone and 6-methyl-3-heptanone, are considered lipids). The 8-methyl-2-nonanone could be found in traces in these samples, while the shorter 6-methyl-3-heptanone was absent. Quantification of the absolute abundance of each ketone in a sample was beyond the scope of this study.

Tests for Biological Activity of Potential Pheromones

Release of male courtship

Male *A. aperta* displayed 100% courtship in the presence of higher concentrations ($\geq 1 \times 10^{-1}$ mg/ml solvent) of 8-methyl-2-nonanone. The difference in male response between the control treatment of the solvent alone and synthetic 8-methyl-2-nonanone at a

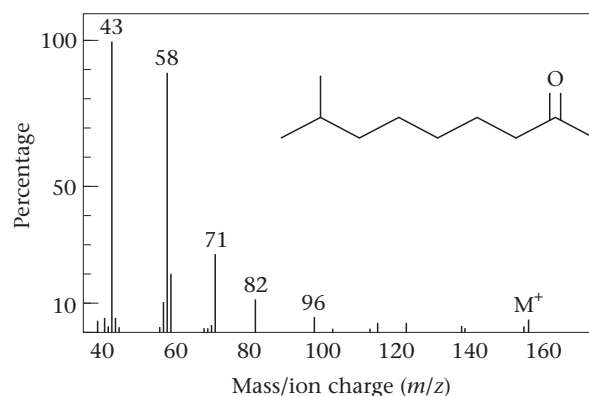


Figure 3. Mass spectrum of 8-methyl-2-nonanone. M^+ indicates molecular ion, mass of 156 unified atomic mass units.

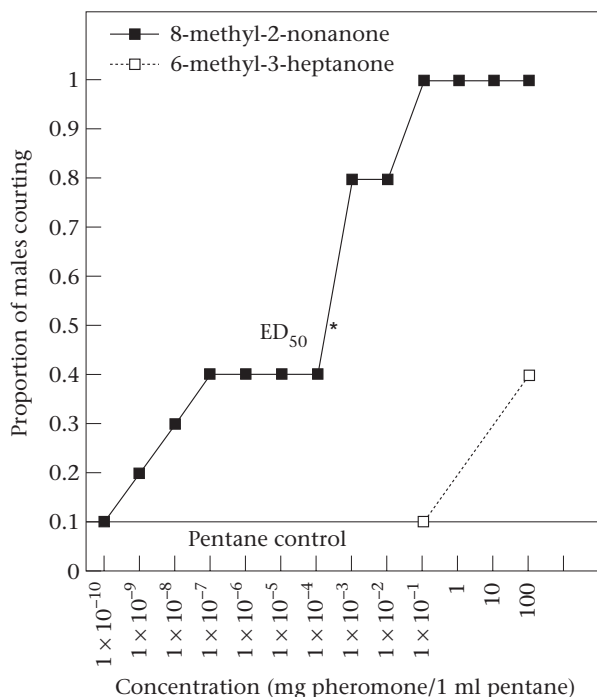


Figure 4. Dose–response curves for male *A. aperta* courtship activity displayed towards two synthesized compounds based on extracts collected from sexually mature female *A. aperta*. Responses of males ($N=31$) to the solvent (pentane) alone are also shown. A minimum of 10 males were tested for each concentration of the two test compounds in pentane.

concentration of 1×10^{-2} mg/ml solvent was highly significant (chi-square test: $\chi^2_1=12$, $P<0.001$). After Carlson et al. (1998), the dose–response data showed an ED_{50} (effective dose index: Pschyrembel 1994) for 8-methyl-2-nonanone at 5.5×10^{-4} mg nonanone/ml solvent (Fig. 4). This is the empirically determined concentration at which 50% of the population of *A. aperta* males is expected to display elements of courtship behaviour. We subjected the dose–response data to a SAS (1995) logistic regression (probit function link) to test for the existence of a dose–response relationship. The slope of the regression between nonanone concentration and proportion of males courting was significant ($\chi^2_1=7.1$, $P<0.008$), indicating that this relationship indeed exists between nonanone concentration and the probability of male courtship. Furthermore, the probit estimate of ED_{50} was found to be 6.5×10^{-4} mg nonanone/ml solvent, a close approximation to the empirically determined value listed above.

At even the highest concentrations tested (100 mg of heptanone/ml solvent), 6-methyl-3-heptanone failed to elicit courtship above the ED_{50} standard (Fig. 4). The frequency of male courtship in the nonanone trials at this high concentration was significantly greater than that elicited in the heptanone trials at the same concentration ($\chi^2_1=6.7$, $P<0.01$). A compound blend of 1×10^{-9} mg of nonanone: 1×10^{-1} mg heptanone failed to have a synergistic or enhancing effect in eliciting male courtship.

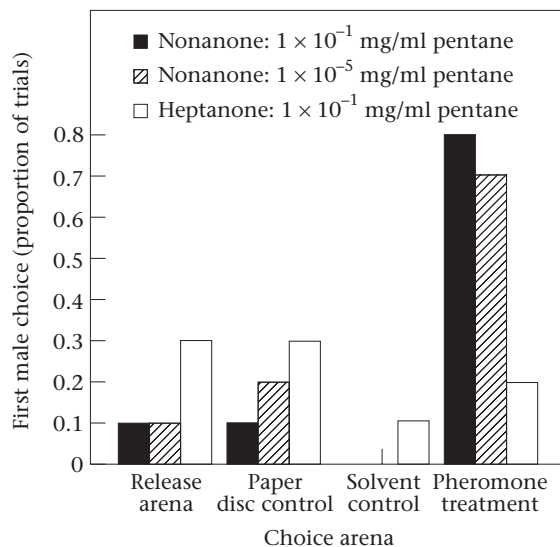


Figure 5. Results of male *A. aperta* attraction to high and low concentrations of 8-methyl-2-nonanone, and high concentrations of 6-methyl-3-heptanone. Trials were completed in three-choice arena system (see Fig. 1). The category ‘release arena’ refers to males that within the 2-h time limit of a trial failed to leave the central arena into which they had been introduced at the start of the trial.

Only one of the 10 males tested displayed elements of courtship behaviour in the presence of this pheromone blend.

Attraction of males

The results of the three-choice arena trials for the attraction effects of the two compounds identified in 2-week postmaturation virgin females are presented in Fig. 5. Male *A. aperta* significantly moved from the release arena directly into the arena containing 8-methyl-2-nonanone at both high (1×10^{-1} mg) and low (1×10^{-5} mg) concentrations of the pheromone (chi-square test: $\chi^2_1=13.5$, $P<0.001$; $\chi^2_1=3.9$, $P<0.05$; Fig. 5). Males showed no choice for a particular arena in the trials involving 6-methyl-3-heptanone at the high concentration of 1×10^{-1} mg heptanone/ml solvent ($\chi^2_1=0.47$, NS; Fig. 5).

Influence on courtship sequence

We used the following courtship sequence parameters as general indicators of level of activity: latency to start courtship, total duration of courtship acts displayed, the frequency of pauses in male courtship activity, and pause length. Only the latency to initiate courtship following introduction of a male test subject showed a significant difference between the treatments: (1) pheromone (8-methyl-2-nonanone) and (2) female *A. aperta*. Males in the pheromone treatment initiated courtship far more quickly than did males in the presence of females (Table 2). This may reflect the fact that the concentration of pheromone applied in the pheromone treatment exceeds what female *A. aperta* release (an extraordinary sign stimulus). An alternative hypothesis would be that males are hesitant to initiate courtship in the presence of

Table 2. Comparison of level of activity displayed in different courtship contexts

Category	Pheromone		Female		Pairwise statistics		
	Mean	(SE)	Mean	(SE)	<i>t</i> value	<i>df</i>	<i>P</i>
Latency to start (s)	224.3	(57.0)	1327.4	(343.9)	3.16	30	<0.004
Total act duration (s)	304.3	(56.9)	200.9	(50.2)	0.41	30	<0.69
Pause frequency	10.9	(1.7)	8.1	(2.0)	1.05	30	<0.30
Mean pause duration (s)	82.0	(38.8)	19.6	(3.0)	1.26	24	<0.22

females of large mass. In the latter case, a male might wait at the web edge where it could make a quick withdrawal if the female were to attack. We tested this latter hypothesis by completing a multiple regression of the latency to initiate courtship as a function of absolute female body mass, absolute male body mass, and the ratio of male mass to female mass. Only the ratio of male mass to female mass showed a significant influence on lag time (F ratio=7.5, $P<0.02$) and was included in the model. The regression relationship between male to female body mass and latency to initiate courtship was $Y = -2816.7X + 4408.0$, with $r^2 = 0.43$.

As indicated by the lack of significant differences between total courtship act duration between the two treatments, a male courted in the presence of a pheromonal cue alone just as actively as in the presence of the female with the additional sensory input she might provide. Only one male in the 32 trials (16 of each class) stopped courting within the 15-min cutoff period. This was a male in the pheromone treatment class.

Significant differences were observed in the behaviour patterns displayed by males in the two treatments. Most pronounced was behaviour recorded in the detection and locating phase of the male courtship sequence (Level 1, Fig. 6). An analysis of variance (ANOVA) completed on male behaviour on this level detected significant behaviour pattern, treatment and treatment by behaviour pattern interaction effects (Table 3). Analysis of variance contrasts identified the higher level of searching by males subjected to the pheromone cue as the major contributing factor to treatment differences (t ratio=4.69, $P<0.00001$). No other behaviour patterns at this level showed significant between-treatment differences.

There was also a significant whole model result observed in the ANOVA completed on Level 2 behaviour by males. This was the signalling phase of the courtship sequence, and here the significant effect was the interaction between treatment and behaviour pattern (Table 3). Analysis of variance contrasts revealed that males in the pheromone trials displayed higher levels of palpating the web with the front legs (t ratio=2.1, $P<0.04$), while far greater levels of web flex were displayed by males in the presence of females (t ratio=4.03, $P<0.0001$; Fig. 6).

Analysis of variance completed on male courtship at the contact phase of the sequence (Level 3), identified significant treatment and behaviour pattern effects (Table 3). Males performed some behaviour patterns in the female-present treatment that they did not perform in the pheromone treatment: retreat, wave legs and attack

(Fig. 6). These behaviour patterns were displayed with low frequency and hence duration. The two behaviour patterns most frequently displayed at Level 3, however, were orient to and approach disc or female. The higher frequency of approaches to the female as opposed to the disc was the significant contributor to the treatment effect noted (t ratio=3.62; $P<0.0004$; Fig. 6).

There was no significant between-treatment effect in the self-preparation behaviour patterns, groom or preen and vibrate abdomen (Level 4) of the courtship sequence. Preening of the palps and legs occurred more frequently than abdomen vibration and thus were of longer total duration in the courtship sequence (Fig. 6). This contributed to a significant behaviour pattern effect and overall significant whole model outcome (Table 3).

DISCUSSION

Pheromone versus Chemical Cue

We have shown that 8-methyl-2-nonanone ('nonanone') is a sex pheromone of *A. aperta*. We use pheromone rather than chemical cue here as this stimulus is obviously adaptive for the sender and produced for the recruitment of males. Williams (1992) argues that many sex pheromones do not fit the definition of a true pheromone in the sense of Wilson (1975) because both sexes and even juveniles frequently emit these substances, although in lesser quantities than those emitted by females. This is not the case with *A. aperta*. Nonanone was collected only from adult virgin females that were sexually mature (receptive), using two detection methods (abdomen wipes and headspace extracts). This does not mean that the use of this chemical in the recruitment of males might not have evolved from another function. This methyl ketone is, to the best of our knowledge, so far known only from the essential oil of *Ruta angustifolia* (Joulain et al. 1991) and hop (Tressl & Friese 1978). Surprisingly, it is the most active synthetic attractant for the mosquito *Culex pipiens* among 124 compounds tested (Ikeshoji & Mulla 1977). Perhaps the ketone was first used by *A. aperta* to attract prey, but its absence in other classes of *A. aperta* argues against this hypothesis. However, a number of gains and losses of given compounds have been reported in the evolutionary history of other arthropods (e.g. Lepidoptera: Tortricidae: Newcomb & Gleason 1998).

Because spiders are carnivorous, the pheromonal ketone, resembling typical natural scent compounds, is

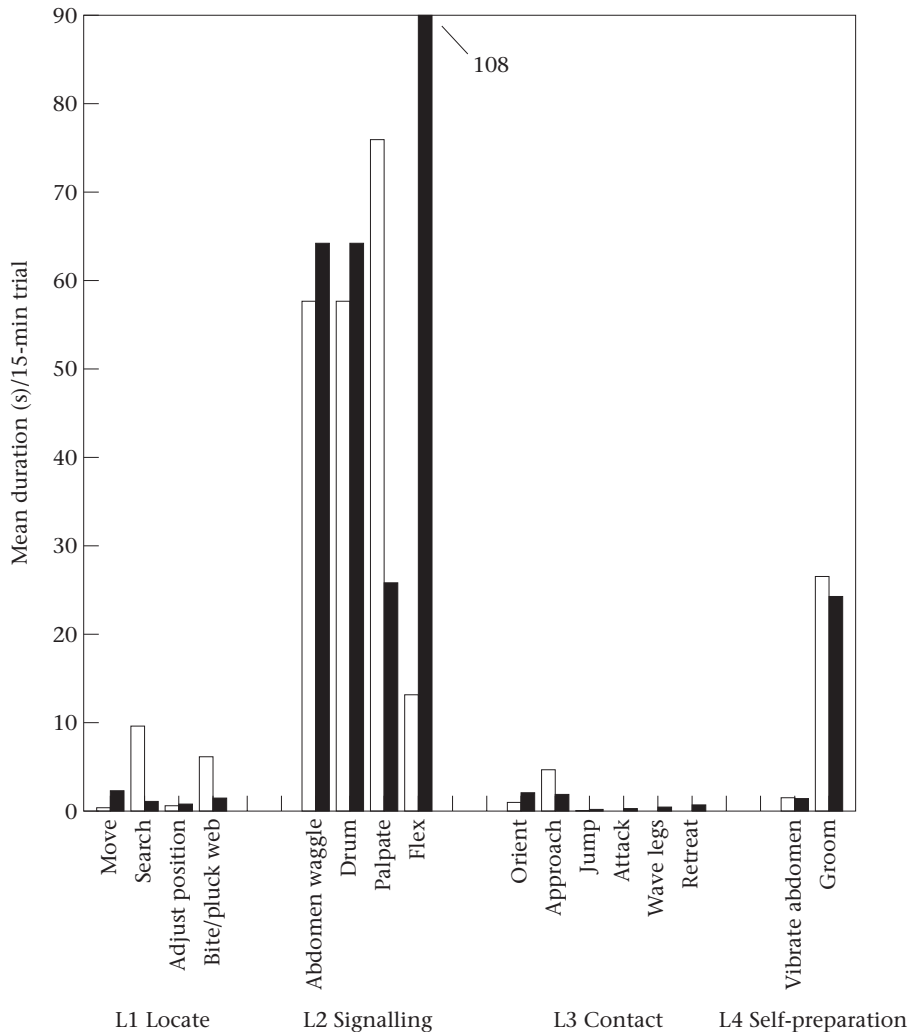


Figure 6. Comparison of male *A. aperta* behaviour displayed in the presence of female *A. aperta* (■) and in the presence of 0.1 mg nonanone/ml pentane applied to a filter-paper disc (□). Means presented are for 16 trials/treatment. Trials were ended 15 min after initiation of Level 2 courtship behaviour (see Table 1 for descriptions of behaviour patterns for each level of the courtship sequence).

not likely to be taken up with feeding. Instead, the compound probably is biosynthesized de novo by starting with 3-methylbutyrate, derived from leucine, followed by typical fatty acid biosynthesis (e.g. Charlton & Roelofs 1991). Chain elongation then would form a 3-oxo-9-methyldecanoate intermediate, which after loss of the carboxyl group furnishes 8-methyl-2-nonanone.

Behavioural Effects

Attraction of male spiders

This study confirms the bioassay test findings of Riechert & Singer (1975) for *A. aperta* and most recently of Rypstra & Persons (1999) for the wolf spider *Parodsa milvina*: females can use volatile pheromones to attract males. Airborne pheromone attraction has been shown for insects in nearly 1000 cases (Hwang 1997), but not for spiders. Earlier studies with spider chemical communication tested for tactile cues associated primarily with silk (see reviews in Tietjen & Rovner 1982; Pollard et al.

1987). The female pheromone in *A. aperta* is manipulative in that the attraction is limited to females in a narrow physiological state, sexually mature and virgin. Although Rypstra & Persons (1999) did not partition their mature test females into nonvirgin, newly matured virgins and sexually mature virgins, male wolf spiders chose virgin females over juvenile (penultimate instar) females and adult males. We observed the same result in *A. aperta*. In both systems, then, males do not visit females at times when they might be potential competitors or predators.

As already mentioned, 8-methyl-2-nonanone appears to be emitted only by females that are sexually receptive. Two lines of evidence suggest that sexual maturity may not be equated with completion of the final moult in this spider: (1) males are not attracted to and fail to court newly matured females (Riechert & Singer 1995), and (2) we failed to collect 8-methyl-2-nonanone from females that were younger than 2-weeks past the last moult. It is not uncommon in insects for sexual maturity to lag behind adult status in females. In the roach, *Eurycotis*

Table 3. Analysis of variance results for comparison of male behaviour in trials involving sexually mature virgin females versus trials in which the pheromone 8-methyl-2-nonanone was provided on a paper disc in a concentration of 0.1 mg pheromone/ml solvent

Source	df	SS	F ratio	P
Level 1: Detection/location				
Whole model				
Model	7	1093.3	5.95	<0.00001
Error	120	3150.6		
Total	127	4244.0		
Effect test				
Behaviour pattern	3	418.9	5.32	<0.002
Treatment	1	171.1	6.52	<0.001
Treatment*behaviour pattern	3	503.3	6.39	<0.0005
Level 2: Signalling				
Whole model				
Model	7	103530.7	3030	<0.003
Error	120	537607.7		
Total	127	641138.4		
Effect test				
Behaviour pattern	3	2159.3	0.16	<0.95
Treatment	1	746.0	0.17	<0.70
Treatment*behaviour pattern	3	100625.5	7.49	<0.0001
Level 3: Contact				
Whole model				
Model	11	347.5	6.54	<0.00001
Error	180	869.8		
Total	191	1217.3		
Effect test				
Behaviour pattern	1	36.8	7.60	<0.007
Treatment	5	269.8	11.17	<0.00001
Treatment*behaviour pattern	5	41.0	1.70	<0.15
Level 4: Self-preparation				
Whole model				
Model	3	9256.6	2.77	<0.05
Error	60	66893.1		
Total	63	76149.8		
Effect test				
Behaviour pattern	1	9216.0	8.27	<0.006
Treatment	1	22.6	0.02	<0.90
Treatment*behaviour pattern	1	18.1	0.02	<0.90

floridana, females are first sexually receptive at 18 days after becoming adults (Farine et al. 1996), although in most cases receptivity increases during the first 0–3 days following attainment of adult status in female insects.

Agelenids have first-male sperm precedence, a consequence of the female's dual opening tube system: the first sperm introduced is thus the first sperm to inseminate the eggs. The sperm a male *A. aperta* provides a female is more than adequate to fertilize her entire reproductive output, consisting of several clutches of eggs in the laboratory rearing environment, but only one to two clutches in the field (S. E. Riechert, unpublished data). The first-male sperm precedence pattern may explain the fact that pheromone production subsides following insemination. Mating is potentially costly to females as mating spiders are nonvigilant towards predators.

No attempt was made in this study to establish the distance at which males will initiate an approach to a female releasing pheromone. However, we detected no drop-off in direct male movement into the chamber containing the pheromone cue between the use of a pheromone concentration above the ED₅₀ that elicits a

100% courtship response and a concentration that was well below the ED₅₀ for release of courtship behaviour. These concentrations are similar to those released by female lepidopterans, which are thought to attract males at distances of 0.1 km (K. Haynes, personal communication).

Courtship Behaviour

The female pheromone 8-methyl-2-nonanone also elicits or releases courtship behaviour in male *A. aperta*. From the dose–response curve, it is clear that this is a strong signal with only minute amounts of pheromone required to stimulate the appropriate male response. The other ketone collected, 6-methyl-3-heptanone, elicited some courting behaviour in males. The frequency of courtship elicited by this latter compound was below the 50% cutoff applied in pheromone trials. As many insect pheromones consist of a blend of active compounds, we did test for a synergistic effect of a blend of a low concentration of nonanone and a high concentration of heptanone. We did not find an enhancing effect of

heptanone on the courtship-releasing action of the nonanone in this particular mixture. It is possible that other proportions of both ketones might show an enhanced effect of the resulting blend compared to the pure nonanone. Discrete blends of sex pheromone compounds frequently have the highest male attraction rate in the Lepidoptera. The determination of an optimized blend for both ketones is outside the scope of this study, and up to now, we do not have any hint of the existence of such a blend. The low response to the heptanone might originate in some structural similarities (same branching pattern of the hydrocarbon chain, ketone functionality) to the real pheromone 8-methyl-2-nonanone. At this point, we have no evidence that the heptanone functions as a pheromone in the *A. aperta* communication system.

The female pheromone 8-methyl-2-nonanone was found to release sexual activity in males that comprised most of the behaviour patterns released by a sexually active female *A. aperta*, and no differences were detected in the total number of courtship acts displayed by males in sequences with the pheromone cue presented alone or in the presence of a sexually mature female. In fact, males initiated courtship activity more quickly towards the pheromone cue alone than they did in the presence of a female. The fact that male initiation of courting in the female-present trials was significantly delayed in cases where the male had a size disadvantage relative to the female, suggests that the concentration of the pheromone used did not present an extraordinary sign stimulus. Rather, female load on the web is an antagonistic stimulus to the attractant stimulus of the female sex pheromone. The pause may well be strategic as males can more readily escape a female attack from the edge of the web.

Inspection of the behaviour patterns displayed within the two treatments (pheromone and female present) identifies two obvious differences and only one unexpected significant difference. These must be discussed in the context of courtship sequence structure in these relatively long and complex courting bouts. From Singer et al. (2000), the general courtship sequence starts with detection and location (Level 1). There follows a prolonged period of male vibratory signalling (Level 2) that leads to male approach and interactive communication with the female (Level 3). Often behaviour patterns in Level 2 are interspersed with those of Level 3. The final stage of courtship (Level 4) is termed mating preparation, as the male's insertion apparatus (palpal organ on front pair of accessory legs) is stimulated through self-grooming and perhaps abdomen vibration, which appear to increase in frequency at this time in the sequence. Because our trials were limited in duration to 15-min following the first male courtship activity, Level 5 behaviour patterns such as male-induced fainting of the female (catilepsis), manipulation of female position, and eventual insertion and insemination were not observed.

Behaviour patterns that were missing in the pheromone-alone treatment included primarily those that might be displayed in response to a female's action, including retreat after female approach or attack, and wave legs, which occurs within visual range (i.e. 2 cm) of

a conspecific. The behaviour pattern termed flex or web bouncing also occurred far more prominently in the female-present treatments than in the pheromone-alone treatments. This behaviour is one of several highly repetitive action patterns associated with what we term the signalling phase (Level 2) of the courtship sequence. It is unclear why there was both a greater frequency and longer duration of male flexing behaviour in the female-present sequences. Male flexing may be cued by female load on the web, although no relationship was detected in the mass of females and the degree of flexing shown by males. A second possibility is that flexing is a type of behaviour that is more readily expressed in response to actions by the female. A sequential analysis of *A. aperta* courtship presented in Singer et al. (2000) indicates that male flex significantly follows female retreat.

In the pheromone treatment, males displayed significantly more actions associated with locating the female (e.g. search in Level 1 or the detection and locating phase, and palpating the web with the front legs in the signalling phase of Level 2). Typically, search and palpating behaviour patterns are of low frequency in courtship sequences (Singer et al. 2000) as *A. aperta* uses vibratory and web-load cues with facility on its sheet-web (Riechert 1984). Even subtle moves by only one of two *A. aperta* spiders on a given web, can provide each individual information about the location and the relative mass of its conspecific (Riechert 1984).

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