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Molecular diagnostics reveal spiders that exploit prey vibrational signals used in sexual communication

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Abstract

Vibrational signalling is a widespread form of animal communication and, in the form of sexual communication, has been generally regarded as inherently short-range and a private communication channel, free from eavesdropping by generalist predators. A combination of fieldwork and laboratory experiments was used to test the hypothesis that predators can intercept and exploit such signals. First, we developed and characterized PCR primers specific for leafhoppers of the genus *Aphrodes* and specifically for the species *Aphrodes makarovi*. Spiders were collected from sites where leafhoppers were present and screened with these primers to establish which spider species were significant predators of this species during the mating period of these leafhoppers. Analysis using PCR of the gut contents of tangle-web spiders, *Enoplognatha ovata* (Theridiidae), showed that they consume leafhoppers in the field at a greater rate when signalling adults were present than when nymphs were dominant, suggesting that the spiders were using these vibrations signals to find their prey. Playback and microcosm experiments then showed that *E. ovata* can use the vibrational signals of male leafhoppers as a cue during foraging and, as a result, killed significantly more male than female *A. makarovi*. Our results show, for the first time, that arthropod predators can exploit prey vibrational communication to obtain information about prey availability and use this information to locate and capture prey. This may be a widespread mechanism for prey location, one that is likely to be a major unrecognized driver of evolution in both predators and prey.

Keywords: eavesdropping, generalist predators, gut content analysis, leafhoppers, predator–prey interactions, vibrational communication

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Introduction

Molecular diagnostics are a powerful tool for measuring ecological interactions in the field, particularly predator–prey interactions between invertebrates (reviewed in Symondson 2002; King *et al.* 2008). Such interactions frequently take place within thick vegetation, often at night, or may be beneath the soil surface or within aquatic environments. Predators are collected and their gut contents analysed, revealing trophic interactions directly in the field under natural conditions. Such PCR-based analyses are equally effective whether the predators chew their prey, and consume everything, or simply ingest fluids, as is the case for spiders and bugs. The tests are, therefore, objective and free from reliance upon morphological identification of consumed remains. This approach is being used to build sophisticated food webs that describe the complexity of interactions occurring within invertebrate communities (Bell *et al.* 2010). Gut analyses have been used in a number of applied field studies involving spiders, mainly as

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potential biocontrol agents feeding on pests in agricultural crops (e.g. Agustí *et al.* 2003; Fournier *et al.* 2008; Kuusk *et al.* 2008; Lundgren *et al.* 2009; Kuusk & Ekbom 2010; Monzo *et al.* 2010).

Here, however, we have used molecular diagnostics in a novel and fundamental way to help us in a study of the mechanisms that predators use to find their prey, in particular the role of vibrational communication signals. Animals have evolved a range of sophisticated chemical, visual and mechanical (air-borne sound and substrate-borne vibrations) signals for intraspecific communication (Bradbury & Vehrencamp 1998; Greenfield 2002). In particular, sexual signals used in pair formation are species- and sex-specific, and are directed at conspecific members of the opposite sex (intended receivers) that use them for recognition (Zuk & Kolluru 1998; Haynes & Yeargan 1999). Furthermore, they also influence the behaviour of the intended receivers in predictable ways, such as movement towards the stationary sender during the processes of localization. Such signals, that stand out from background 'noise' and are rich in information, may also be intercepted by predators in the same habitat (Burk 1982; Lima & Dill 1990; Magnhagen 1991; Verrell 1991; Zuk & Kolluru 1998; Haynes & Yeargan 1999). Such exploitation, by predators, of chemical, visual and air-borne sound communication has been well documented (Zuk & Kolluru 1998; Haynes & Yeargan 1999; Lewis & Cratsley 2008). Substrate-borne vibrational communication has, by contrast, been considered to be a channel not readily accessible to generalist predators (Römer *et al.* 2010). Indeed, it is often regarded as an adaptation for avoiding detection by enemies (Belwood & Morris 1987; Lima & Dill 1990; Henry 1994; Zuk & Kolluru 1998; Cooley 2001; Lang *et al.* 2005), potentially accessible only to specialized parasitoids (Laumann *et al.* 2007). It seems unlikely that predators would ignore vibrational signals as a potential source of information, helping them to find their prey. Many groups of organisms use vibrational signals (Hill 2008), especially insects (Virant-Doberlet & Čokl 2004; Cocroft & Rodríguez 2005). In spiders, sensory guided behaviours such as prey detection and intraspecific communication are commonly mediated by vibrational signals (Barth 1998; Uetz & Roberts 2002). Furthermore, arthropod predators have a variety of specialized and highly sensitive receptors to detect substrate vibrations (Barth 1998, 2004; Brownell & van Hemmen 2001). Vibrational signals incorporated within multimodal courtship displays, which include both vibrational and visual signals, increase detectability by predators (Roberts *et al.* 2007). Furthermore, unintended receivers are known to exploit incidental vibrational cues induced by walking or feeding prey (Pfannenstiel *et al.* 1995; Barth 1998; Meyhöfer & Casas 1999; Brownell & van Hemmen 2001). Indeed, such cues are generally less conspicuous and less informative than the signals used in sexual communication.

The plant-dwelling Auchenorrhyncha (leafhoppers, planthoppers, froghoppers and treehoppers) are the dominant macroarthropods (by number and species richness) in many grasslands and are therefore an important prey source for higher trophic levels (Biedermann *et al.* 2005). Sexual communication (mate recognition and location) in these insects is mediated exclusively by species- and sex-specific vibrational signals (Claridge 1985; Čokl & Virant-Doberlet 2003). The male and female establish a duet and then the male approaches the stationary female using her replies as a cue for localization. Spiders, the most numerous group of generalist predators above ground in many terrestrial habitats (Wise 1993), are major predators of leaf- and planthoppers (Döbel & Denno 1994; Fournier *et al.* 2008; Sanders *et al.* 2008), and wolf spiders (Lycosidae) are usually considered as the most important predator group (Döbel & Denno 1994; Preap *et al.* 2001; Widiarta *et al.* 2001). There is also some anecdotal evidence that jumping spiders (Salticidae) may use leafhopper vibrational signals to locate their prey (Narhardiyati & Bailey 2005). We studied interactions between a natural assemblage of spiders and, as prey, the leafhopper *Aphrodes makarovi* (Hemiptera: Cicadomorpha: Cicadellidae), inhabiting interconnected patches of vegetation along the borders of a meadow.

We tested the hypothesis that generalist predators (spiders) can exploit insect vibrational communication. First, we used PCR to determine which species of spider are major predators of *A. makarovi* in the field and to establish that predation by spiders, during the mating period of these leafhoppers, occurs frequently enough to be a significant source of mortality. Two contrasting species of spiders, which were shown in the molecular analysis of field-caught spiders to be either major predators or non-predators of *A. makarovi*, were chosen for further behavioural experiments. We assessed, in microcosms, the impact of these two spider species on leafhopper mortality and selection of prey by sex. We used playback experiments to test whether spiders change their behaviour in the presence of *A. makarovi* vibrational signals. We used molecular diagnostics to test the hypothesis that rates of predation by spiders on leafhoppers would be greater when adult, sexually communicating, leafhoppers were present in the field compared with non-signalling juveniles.

Materials and methods

Field site and sampling

The field site was in Lisvane, Cardiff, UK (N: 51°32.160′; W: 03°10.173′), where the vegetation was dominated by

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stinging nettle (*Urtica dioica*), bindweed (*Convolvulus arvensis*) and bramble (*Rubus fruticosus*). The presence of *A. makarovi* at the site (rather than another species of *Aphrodes*) was determined by recording the vibrational signals of 11 males using a laser vibrometer (PDV 100; Polytec GmbH, Waldbronn, Germany) (e.g. Mazzoni *et al.* 2009) and comparing them with known signals (Tishechkin 1998). This was necessary because there is considerable overlap in the morphological characters used to separate species in the genus *Aphodes*. At each sampling date, we visually checked for the presence of *Aphrodes* leafhoppers (nymphs and/or adults). Spiders were collected from the foliage either by hand or by a motor-driven suction sampler (McCulloch, BVM 250; Electrolux) using a sampling cylinder with a 11.5 cm diameter. Although vacuum sampling methods have been frequently used for collecting spiders, this method can lead to external contamination by prey DNA that can increase the risk of false positives when predators are screened with molecular methods (King *et al.* 2008; Greenstone *et al.* 2011). In particular, this is important when dealing with soft, fragile prey like aphids that can be damaged in the suction sampler. In addition, when the suction is turned off, spiders may simply catch and feed on the nearest prey. To minimize the risk of cross-contamination, we sampled for short intervals (60 s) and spiders were taken from the net immediately and stored in ethanol. Furthermore, we never observed spiders in the net catching *Aphrodes* leafhoppers. While adult *Aphrodes* leafhoppers have hard cuticle, nymphs are soft-bodied. External contamination would therefore be expected to be higher when only nymphs were present, and false positives would be detected in all spider groups collected at the time. None of these predictions was confirmed when the field data were analysed (see Results).

Only spiders considered large enough to kill *Aphrodes* nymphs or adults (body length ≥4 mm) were collected and were immediately transferred to separate 7-mL vials (Greiner bio-one, Stonehouse, UK) of 100% ethanol. Once back in the laboratory, the vials were stored at −80 °C until DNA extraction. All spiders were identified, and the number, stage and gender of *Aphrodes* collected on each sampling date were recorded.

Molecular analysis of predation in feeding trials and in the field

Primer design. The target prey were leafhoppers of the genus *Aphrodes* (Hemiptera: Cicadellidae). However, to design primers, sequences needed to be obtained from related Hemiptera to allow target-specific primer sites to be identified. Up to ten individuals of Cicadellidae (*Aphrodes makarovi*, *A. bicincta*, *Cicadela viridis*, *Cicadulla quadrinotata*, *Conosanus obsoletus* and *Macrosteles* sp.) and Cercopidae (*Aphrophora alni* and *Philaenus spumarius*) collected at various locations around Cardiff (Wales, UK) were sequenced. To include all recognized *Aphrodes* species in Europe, specimens of *A. diminuta* and *A. aestuarina* were collected in Sussex at Castle Hill, on the South Downs, and at Shoreham, respectively. The predators, too, needed to be sequenced to ensure that any *Aphrodes* primers that were designed did not co-amplify spider DNA. Sequences were therefore obtained from members of the Salticidae (*Heliophanus flavipes*), Thomisidae (*Xysticus cristatus*), Araneidae (*Araneus quadratus*) and Linyphiidae (*Linyphia triangularis*). Ethanol-preserved specimens of insects and spiders used in primer design were stored at −20 °C until DNA extraction. For insects, either whole body (smaller species) or all six legs (larger species) were used for DNA extraction. Spiders were starved for 10 days, and only the tarsal segments of six legs were used for DNA extraction because gut diverticula extend into other segments of spider legs. Because tarsal structures are also used as systematic characters, two legs were left intact. DNA was extracted using DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's spin-column protocol.

A 710-bp fragment of the mitochondrial cytochrome oxidase subunit I gene was amplified with the universal primers LCO1490 and HCO2198 (Folmer *et al.* 1994) using a BioRad DNA Engine PTC 200 thermal cycler. Polymerase chain reactions (PCR) were carried out in total volumes of 25 μL consisting of 10× PCR buffer (Invitrogen, Paisley, UK), 4 mM $MgCl_2$ (Invitrogen), 0.1 mM each dNTP (Invitrogen), 2.5 μg bovine serum albumin (BSA; 10 mg/mL; New England Biolabs, Hitchin, UK), 0.2 μM each forward and reverse primers, 0.625 U Taq DNA polymerase (Invitrogen) and 2.5 μL DNA (1:5 dilution of original extract). PCR cycling conditions used were 94 °C for 2 min 30 s, followed by 35 cycles of 94 °C for 30 s, 47 °C for 30 s, 72 °C for 40 s and a final cycle of 72 °C for 10 min. PCR products (10 μL) were purified using 0.25 U each of Exonuclease I (New England Bioloabs) and Shrimp Alkaline Phosphatase (USB) incubated at 37 °C for 45 min and 80 °C for 15 min. Products were sequenced directly using the original forward and reverse PCR primers on a 3130xl Genetic Analyser (Applied Biosystems) using a BigDye (version 1.1) sequencing kit. Sequence alignment and editing were carried out using Sequencher version 3.1.2 (Gene Codes), and secondary alignment was carried out by Clustal (Thompson *et al.* 1997) using default settings (gap opening 15 and gap extension penalties 6.6666). To incorporate the extent of variation within target prey and closely related taxa in the primer design, all haplotypes of *Aphrodes* found were included in the alignments. All sequences were submitted to GenBank

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(accession numbers for all *Aprodes* spp. FR727167-FR727179, *Cicadella viridis* FR775764, *Cicadula quadrinotata* FR775765, *Conosanus obsoletus* FR775766, *Xysticus cristatus* FR775767, *Aphrophora alni* FR775768, *Philaenus spumarius* FR775769, *Heliophanus flavipes* FR775770, *Linyphia triangularis* FR775771, *Araneus quadratus* FR775772 and *Macrosteles* sp. FR775773).

Aphrodes genus-specific and *A. makarovi* species-specific primers were designed using Amplicon software (Jarman 2004). Primer sequences, expected product sizes (bp) and optimal annealing temperatures (°C) are shown in Table 1. Primer specificity was evaluated by running PCR on both target and a broad range of non-target species (Table S1, Supporting information).

Feeding experiments to calibrate post-ingestion detection of prey DNA in predators. Wolf spiders, *Pardosa amentata*, were collected from the Lisvane site by suction sampler in August 2007 and maintained at 16 °C on a 16:8 light:-dark cycle. Spiders were housed separately in plastic containers (diameter 8.5 cm and height 5 cm) containing damp peat and moist tissue to ensure high humidity. They were fed once with live *Drosophila hyde* and hatchlings of *Gryllus bimaculatus* (both from Livefood, Rooks Bridge, UK) and then starved for 2 weeks. On the day of a feeding trial, they were transferred to 35 × 10 mm sterile Petri dishes (Becton Dickinson, Oxford, UK). Adult *Aphrodes* leafhoppers were collected at the same site in July and August 2007 and frozen at −80 °C. On the day of the feeding trial, leafhoppers were cut in half along the body axis and left at room temperature to defrost completely. Spiders were given half of a leafhopper and allowed to feed over a period of 180 min. At the end of a feeding period, 10 unfed wolf spiders were killed as starved controls. Thirty-nine spiders that were observed to feed for at least 60 min were included in the analysis. Five of them were killed in 100% ethanol immediately after the 180-min feeding period. The remaining spiders were transferred into clean plastic containers, kept at 16 °C and killed at 6, 12, 24, 48, 72, 96 and 120 h after the feeding period, with five replicates at each time period (for 120 h, only four spiders were included).

Tangle-web spiders, *Enoplognatha ovata*, were collected from the same Lisvane site by suction sampler in June 2008 and maintained at 20 °C on a 16:8 light:dark cycle. Spiders were housed separately in plastic containers as described earlier. They were fed once with live *D. hyde* and starved for a week. One day before the feeding trial, they were transferred to 35 × 10 mm sterile Petri dishes (Becton Dickinson). Each spider was offered one live *Aphrodes* nymph collected at the Lisvane site and was allowed to feed for 180 min. Forty-four spiders that were observed to feed for at least 90 min were included in the analysis. After the feeding period, six spiders were killed immediately (0 h) and the remainder transferred to clean containers and kept at 20 °C. Six to seven spiders were killed at 6, 12, 24, 48, 72 and 120 h after feeding. Ten unfed spiders were killed as starved controls. All spiders were stored in 100% ethanol at −80 °C until DNA extraction.

Up to 5 days before DNA extraction, spider abdomens were removed under a dissecting microscope. To avoid cross-contamination between spiders, forceps were sterilized by flaming after each individual dissection. Abdomens were transferred into sterile 1.5-mL Eppendorf tubes and kept at −80 °C until DNA extraction. The rest of the body was kept frozen at −80 °C. DNA was extracted from spider abdomens using DNeasy Blood & Tissue kit (Qiagen) following manufacturer's spin-column protocol. Abdomens were first homogenized in extraction buffer using DNA-free pestles. For large spiders like *Pisaura mirabilis* and *Agelena labyrinthica*, abdomens were homogenized in 250 μL tissue culture water (Sigma) and 25 μL of homogenate was used for the DNA extraction. The DNA was resuspended in 200 μL of the manufacturer's elution buffer and stored at −20 °C. To check the success of the DNA extractions, universal primers 16sar and 16sbr (Palumbi *et al.* 1991) were used to amplify a 490-bp fragment of the 16S ribosomal RNA (rRNA) gene. PCRs were carried out in total volumes of 12.5 μL consisting of 10× PCR buffer (Invitrogen), 4 mM $MgCl_2$ (Invitrogen), 0.1 mM each dNTP (Invitrogen), 2.5 μg bovine serum albumin (10 mg/mL; New England Biolabs), 0.2 μM each forward and reverse primer, 0.625 U Taq DNA

Table 1 Primers designed from the cytochrome oxidase subunit I sequence of mitochondrial DNA to detect *Aphrodes* leafhoppers. Primer sequences (5′-3′), size of amplified fragments and optimized annealing temperatures are shown

Target	Primer name	Primer sequence	Fragment size (bp)	Annealing temperature (°C)
Genus *Aphrodes*	APH-COI-F1	TAGATTTATTATTCGTATTGAAC	289	60
	APH-COI-R1	ATAAACAGTTCAACCAGTACCA		
A. makarovi	APH-M-COI-F4	AGGTGCTCCTGATATAGCATTTCCT	348	65
	APH-M-COI-R5	TGCTAAAACAGGTAATGAAAGC		

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polymerase (Invitrogen) and 1 μL DNA. PCR cycling conditions used were 94 °C for 2 min 30 s, followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 45 s and a final cycle of 72 °C for 10 min. PCR products were visualized under UV light on 1.5% agarose gels stained with ethidium bromide.

DNA extractions from spiders were then screened for the presence or absence of *A. makarovi* DNA using both primer pairs but in separate PCR reactions. Optimum amplification was obtained using the Qiagen multiplex kit. Amplifications were performed separately for each primer pair in 10 μL, containing 1 μL of extracted DNA, 5 μL of 2× Multiplex PCR mix, 0.2 μM of each of the primers and 0.2 μg BSA. PCR cycling conditions were 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 60 or 65 °C (annealing temperatures for respective primer pairs) for 90 s, 72 °C for 90 s and a final cycle of 72 °C for 10 min. *Aphrodes* DNA was included in each PCR assay as a positive control. In addition, when field samples were tested, positive samples from feeding trials were also included as additional controls. At least five replicates of tissue culture water were included as negative controls in each PCR run. PCR products were visualized on 1.5% agarose gels stained with ethidium bromide. Each individual obtained from feeding trials and field sampling was screened separately three times with each primer combination, and spiders showing PCR product of the expected size at least once with each primer combination were scored as positive. Only samples that tested positive with both primer sets were deemed positive.

Predation experiments in microcosms

Microcosms were set up in 3-L plastic boxes (19.5 × 14.5 × 15 cm; Really Useful Box, Normanton, UK). Each contained five stinging nettles cuttings (12 cm high) standing in vials of water to prevent withering and secured in an upright position by sinking them in a 3-cm layer of peat. Nettle leaves filled the entire volume of the box. Prior to the experiment spiders were starved for 2 weeks.

Although field-caught funnel-web spiders showed higher predation rates on leafhoppers than tangle-web spiders, their numbers in the field were low and the latter were chosen for further tests. Each microcosm contained 10 *A. makarovi* leafhoppers (five males and five females) and either one adult female tangle-web spider, *E. ovata* (ten replicates), or one adult female wolf spider, *P. amentata* (five replicates). Twenty leafhoppers (10 males and 10 females) and all spiders were measured and weighed before the experiment. In addition, three non-predator control microcosms were included (10 leafhoppers, five males and five females in the absence of a predator). The experiment ran for 8 days. Microcosms were checked daily, and all living leafhoppers in each box counted. Dead leafhoppers were removed (except the ones on which spiders were currently feeding). Each dead leafhopper was inspected under a dissecting microscope to determine the cause of death. The effect of predator species on survival of leafhoppers was determined by comparing numbers of surviving leafhoppers in the two spider treatments with numbers in control microcosms using regression analysis. We assessed the effect of leafhopper sex on predation rates by performing analysis of covariance using a linear regression model.

Playback experiments

Playback experiments were performed in August 2008 with 18 adult female *E. ovata* and 15 adult female *P. amentata*. Spiders were starved for 2 weeks before the playback experiments. Spiders were tested on a bean plant (*Vicia faba*, Sutton Dwarf; height 25 cm, 10 branches) growing in a pot. To facilitate comparisons, and avoid problems of heterogeneity in leaf architecture between plants, all experiments were carried out on the same plant that was cleared of silk threads after each trial. All playback experiments were carried out between 0900 and 1800 hours at 23 ± 1 °C and relative humidity 65 ± 10%. Male *Aphrodes* calling signals, female calling signal and incidental vibrational signals induced by walking *Aphrodes* leafhoppers were used (Fig. S1, Supporting information). In control experiments, spider behaviour was observed in the absence of stimuli. The playback stimuli were randomly chosen from a library of recordings (M. Virant-Doberlet, unpublished data). Three male and three female calling signals were used. Each stimulation programme was composed of one recorded signal played back in a continuous loop with 6 s of silence between signals. For incidental signals, we used three recorded sequences of 5 min duration. Each sequence was played back in a continuous loop for the duration of the test. The apex of one bean leaf was vibrated with the conical tip of a 5-cm aluminium rod (diameter 4 mm) screwed firmly into a mini-shaker head (Brüel & Kjaer, type 4810; Naerum, Denmark), driven from the computer via Cool Edit Pro 2 (Syntrilium Software, Phoenix, USA). Playback signals were recorded from the main bean stem with a laser vibrometer, and their amplitude was adjusted to the level of recorded natural leafhopper signals and actual differences in amplitudes of natural stimulation signals were kept unchanged. Spiders were tested in a 5-day experimental series. On days 1 and 5, all spiders were tested in control trials, with no playback signals. For tests on days 2–4, spiders were divided into three equal groups and,

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on each day, each group was tested with one different type of the stimulus, so that by the end of day 4, each spider was tested with all three different types of leafhopper vibrational signals. The order in which groups were tested differed each day. Each of the three stimulation sequences was randomly assigned to spiders in each group, so that on each day, two spiders in each group were tested with the same vibrational stimulus. Such an experimental design allowed us to check for any potential influence of different levels of hunger and habituation on spider responses over the 5 days. We placed a single spider on the top of the bean plant and allowed it to settle for 2 min in the absence of a stimulus. Then, we applied 13 min of stimulation with one of the *Aphrodes* vibrational signals. We monitored how long spiders stayed on the plant. Following one-way analysis of variance, least significant differences (Fry 1993) was used a priori to compare residence time in treatments with each of the two controls.

Results

Primers and feeding trials

Both primer pairs proved to be specific for their targets, with no co-amplification of DNA from any other invertebrate species tested. Screening of DNA extractions from feeding trials showed that *Aphrodes* DNA could be detected in spiders for up to 120 h (Fig. 1). Regression analysis could not be used to model a median detection time (King *et al.* 2008) in the gut of *P. amentata*, owing to lack of time intervals tested beyond 120 h. However, both primer pairs detected *Aphrodes* DNA in *P. amentata* after 120 h (Fig. 1a). All *E. ovata* spiders tested positive for *Aphrodes* DNA up to 12 h after feeding. The calculated median detection time for *Aphrodes* DNA in the gut of this tangle-web spider was 65.8 and 72.8 h after feeding, when amplified with the *Aphrodes* genus-specific and *A. makarovi* species-specific primer pairs, respectively (Fig. 2). None of the starved spiders, of either species, tested positive.

Screening of field-caught spiders

Spiders representing nine families were collected during two field seasons (Table 2). In both years, tangle-web spiders (Theridiidae) were the dominant taxon because of high numbers of *Enoplognatha ovata* and *E. latimana*. Overall, 14.1% of the spiders tested positive for *Aphrodes* DNA (Table 2). Only tangle-web spiders and funnel-web spiders (Agelenidae) showed consistently high rates of predation on *Aphrodes* leafhoppers over two field seasons. Although wolf spiders of the genus *Pardosa* (Lycosidae) are thought to be significant predators of plant- and leafhoppers (Döbel & Denno 1994; Preap *et al.* 2001; Widiarta *et al.* 2001), none of the 36 individuals tested contained *Aphrodes* DNA.

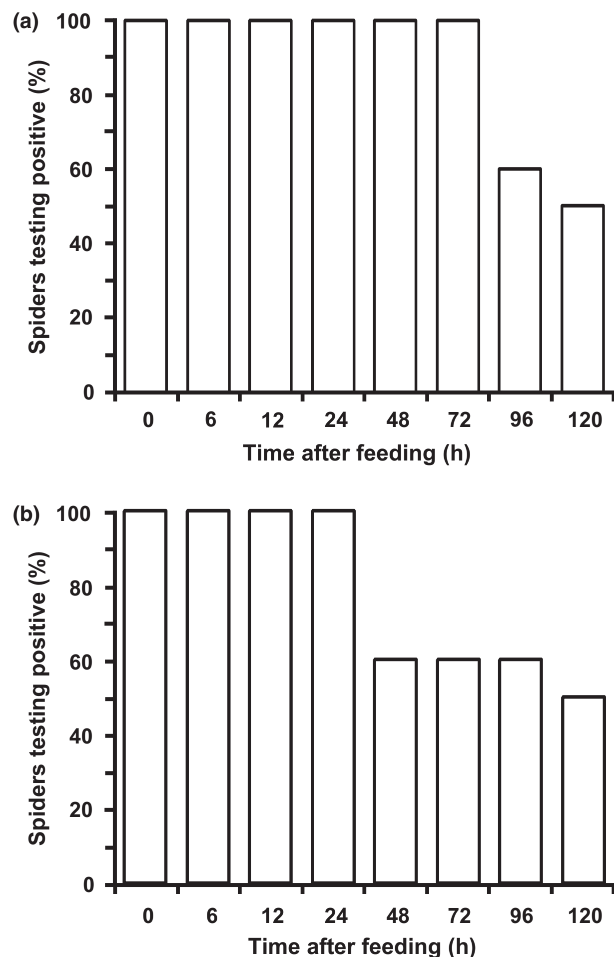

Fig. 1 Percentage of wolf spiders, *Pardosa amentata*, which tested positive for *Aphrodes* DNA 0–120 h after feeding on *Aphrodes* leafhoppers (a) using *Aphrodes* genus-specific primers and (b) using *A. makarovi* specific primers.

The influence of the sampling date on the number of spiders containing *Aphrodes* DNA in their guts was tested on the more numerous tangle-web spiders of the genus *Enoplognatha* in 2008. A significant difference was found between the number of these spiders testing positive on 10 June and 14 July, when 7.1% and 25.5% of tangle-web spiders tested positive, respectively (Fisher's exact test, $P = 0.041$; Fig. 3). The structure of the *Aphrodes* population at the sampling site differed between these two sampling dates. On the first sampling date, only non-signalling nymphs were present; by mid-July, 88.7% of leafhoppers collected were adults (66% males and 22.6% females) that were sexually active. This suggests that during the mating period, when actively signalling to one another, *Aphrodes* leafhoppers face a significant greater risk of predation by *E. ovata*.

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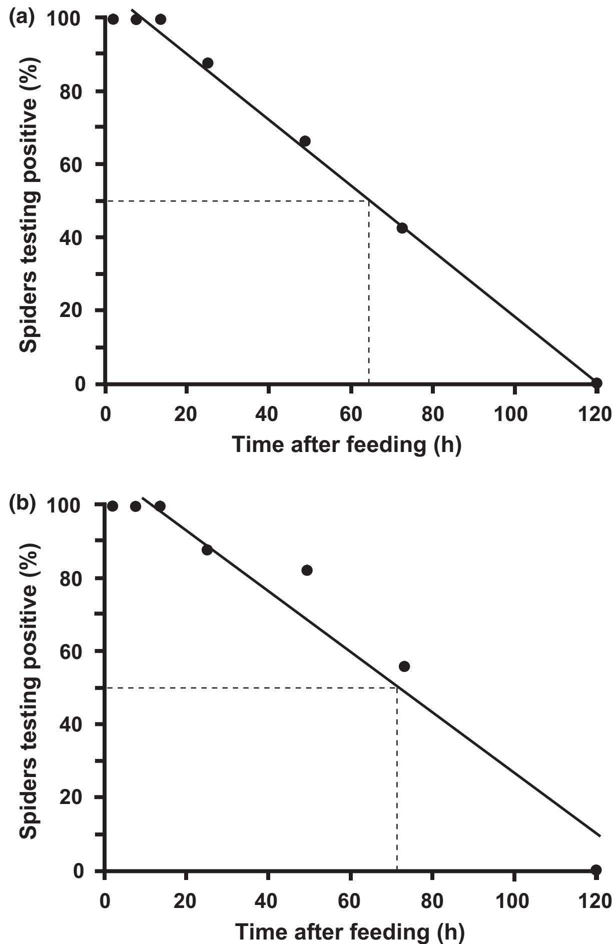

Fig. 2 Detectability of *Aphrodes* DNA in the guts of the tangle-web spider *Enoplognatha ovata* using (a) *Aphrodes* genus-specific and (b) *A. makarovi* species-specific primers. Regression equations were: *Aphrodes* genus-specific primer pair, $y = -0.872x + 107.4$; $R^2 = 0.99$, and *A. makarovi* species-specific primer pair, $y = -0.8204x + 109.7$; $R^2 = 0.94$. The dotted lines indicate the mean detection period calculated from the regression equations.

Microcosm experiments

Although *P. amentata* spiders are significantly bigger than *A. makarovi* (Fig. S2, Supporting information), they had no effect on leafhopper numbers in the microcosms; *A. makarovi* mortality over 7 days was no greater in the presence of the *P. amentata* than in control arenas without predators (mortality was negligible, with neither regression slope for mortality vs. days being significantly different from zero). The mean number of leafhoppers that died in each arena over 1 week, in both the control and *P. amentata* treatments, was exactly one (SEM 0.7 for controls and 0.6 for *P. amentata*), and none of the dead leafhopper in the *P. amentata* treatment was killed or eaten. By contrast, *E. ovata* significantly reduced leafhoppers' numbers over the same period ($F_{1,68} = 211.1$, $R^2 = 75.6$, $P < 0.001$). In the absence of other prey, each tangle-web spider killed around four leafhoppers per week. Furthermore, although the presence of *E. ovata* significantly reduced the numbers of both males and females, the decline in the numbers of males was significantly more rapid (Fig. 4). This indicates that males face a higher predation risk because of either greater detectability (interception of their sexual communication signals) or possibly some other factor, such as higher mobility.

Responses to vibrational signals

Direct evidence that spiders exploit *Aphrodes* vibrational signals to capture prey was obtained by playing pre-recorded vibrational signals of *A. makarovi* to spiders. *E. ovata* changed its behaviour in response to different types of vibrational signals. Because in each treatment, including control trials, few spiders (1–4 individuals) left the plant via the rod connected to the vibration device, there was no significant evidence that *E. ovata* located the source of vibrations. However, when compared with control trials (absence of stimuli), these spiders spent significantly ($P < 0.05$) more time on the plant, but only in the presence of the male calling signal (Fig. 5a). Furthermore, only in this treatment, did we also observed clearly directed movements towards the vibrated leaf and all spiders leaving the plant walked to the mini-shaker. Taken together, these results showed that *E. ovata* spiders changed their foraging behaviour specifically in response to the vibrational signals from male *Aphrodes*. Because sex pheromones are ubiquitous in spiders (Gaskett 2007), we checked for any possible cumulative effects of the pheromone cues of previous spiders tested on the same plant on the same day. All spiders were females, and therefore, no sexual attraction (manifested as increased residence time) was expected, but territoriality could have affected spider responses (either positively or negatively). Linear regression analysis was used with residence time as the response variable and sequential time periods (1–18) as predictors. Analyses were conducted on the data for both controls (days 1 and 5) and test signals (days 2–4) but no significant relationships were found ($P > 0.05$). Wolf spiders did not show any response to playbacks of *Aphrodes* vibrational signals (Fig. 5b).

Any possible effects of habituation or hunger on spider residence times on the plants were tested by comparing controls on days 1 and 5, but no significant effects were found for either *E. ovata* or *P. amentata* ($P > 0.05$, Fig. 5).

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Table 2 Spiders sampled at our Lisvane field site (Cardiff, UK) in 2007 and 2008. The total number of spiders tested in each family (*N*), the number of individuals for each species or genus tested (*n*), and the number and percentage (in brackets) of spiders that tested positive for *Aphrodes* DNA are given

			2007		2008	
Family	*N*	Genus/species	*n*	Positive (%)	*n*	Positive (%)
Clubionidae	12	*Clubiona reclusa*	3	0	1	0
		C. neglecta	–	–	1	0
		Clubiona sp.	–	–	7	1 (14%)
Thomisidae	4	*Xysticus cristatus*	4	1 (25%)	–	–
Lycosidae	36	*Pardosa amentata*	10	0	1	0
		Pardosa prativaga	3	0	–	–
		Pardosa pullata	2	0	–	–
		Pardosa sp.	20	0	–	–
Pisauridae	7	*Pisaura mirabilis*	4	1 (25%)	3	0
Agelenidae	9	*Agelena labyrinthica*	5	4 (80%)	4	2 (50%)
Theridiidae	192	*Enoplognatha ovata*	59	5 (9%)	102	18 (18%)
		E. latimana	11	2 (18%)	9	2 (22%)
		Theridium pictum	11	3 (27%)	–	–
Tatragnathidae	16	*Meta mengei*	9	0	2	0
		M. segmentata	–	–	1	0
		Pachignata clerckii	1	0	–	–
		Tetragnatha montana	–	–	3	0
Araneidae	6	*Larionides cornutus*	3	0	–	–
		Araneus diadematus	1	0	–	–
		Araniella cucurbitina	1	0	–	–
		Agalenatea redii	1	0	–	–
Linyphiidae	1	*Neriene clathrata*	–	–	1	0
Total	283		148	16 (11%)	135	23 (17%)

Discussion

We tested the hypothesis that spiders may be exploiting the vibrational sexual communication signals of their prey to find their prey. Molecular analysis of the gut contents of a range of spider species inhabiting the same field sites as *Aphrodes* leafhoppers showed very clearly that some species, such as wolf spiders, that are usually considered as significant predators of leafhoppers and planthoppers (Döbel & Denno 1994; Preap *et al.* 2001; Widiarta *et al.* 2001), were not feeding on *A. makarovi*. Other spiders, however, including the tangle-web spiders of the genus *Enoplognatha*, were consuming *A. makarovi* at high rates. Most interestingly of all, these spiders were consuming *A. makarovi* at higher rates when adults dominated in the field but at much lower rates when mainly nymphs were present. As only adults produce vibrational signals used in sexual communication, this strongly suggested that *E. ovata* may be exploiting these sexual signals to find their prey, which was explored further with laboratory experiments. Without molecular diagnostics, such information would have been difficult if not impossible to obtain.

Overall, the results provide, for the first time, compelling evidence that generalist arthropod predators can intercept insect vibrational communication to obtain information about prey availability and use that information to locate and capture prey. Predation by *E. ovata* during the mating period for *A. makarovi* occurs frequently enough in the field to be a significant source of mortality, and there is a significant positive relationship between male vibrational signalling and the risk of predation. Furthermore, for *P. amentata*, which was not found to be a predator of this leafhopper species either in the field or in the microcosm experiments, the vibrational signals of *A. makarovi* did not influence spider behaviour.

Because male leafhoppers signal and search, the increased predation risk could result from signalling activity and/or exposure to predators while changing position to increase signalling space (Gwynne 1987; Gwynne & Bussière 2002; Cocroft 2003) and/or during localization of a responding female (Burk 1982; Gwynne 1987). High predation on *A. makarovi* males might simply result from their greater mobility and therefore increased probability of encounters with *E. ovata*. However, if vibrational signals had no influence on spider behaviour, our results would not have shown that male vibrational signals play an important role in site selection. *Enoplognatha ovata* builds a small, inconspicuous,

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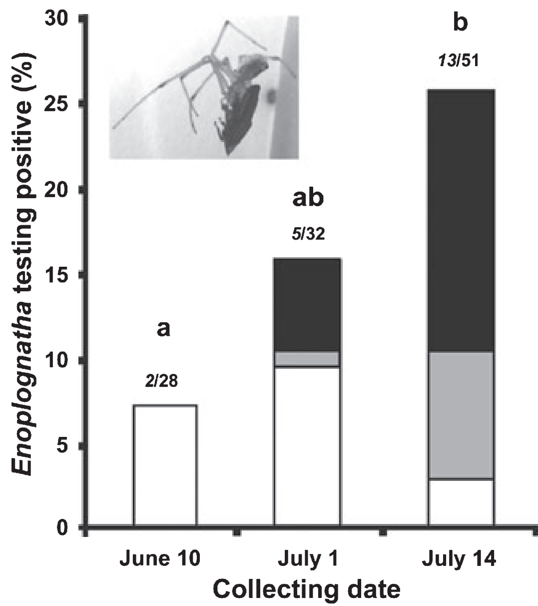

Fig. 3 Percentage of *Enoplognatha* spiders collected in 2008 testing positive for *Aphrodes* DNA. The numbers above bars are numbers of spiders testing positive (in italics) and numbers of spiders collected on each sampling date. Dates with different letters are significantly different for numbers testing positive (Fisher's exact test, $P < 0.05$). Shading in each bar represents the structure of *Aphrodes* population at the sampling site on collection dates. Proportions of nymphs (white), adult females (grey) and adult males (black) are shown. Inset shows *E. ovata* feeding on an *Aphrodes* nymph. Picture was taken during the feeding trials.

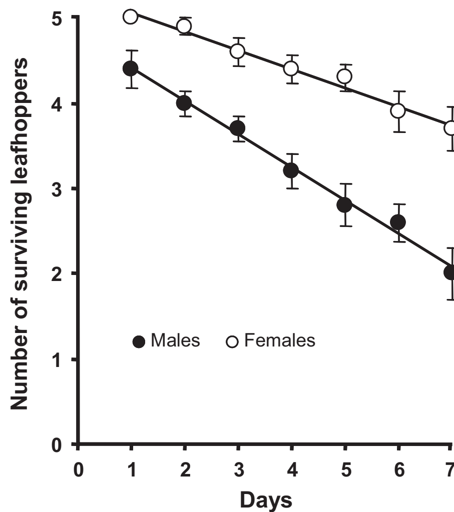

Fig. 4 The effect of *Enoplognatha ovata* spiders on the number of surviving male and female *Aphrodes* leafhoppers. Mean values ± SEM per microcosm (N = 10) are shown. General linear model analysis of covariance (on raw data, not means) gave an overall R^2 of 67.5%, significant slopes for both regression lines ($F_{1,136}$ = 143.43, P < 0.001) and a significant difference between the slopes ($F_{1,136}$ = 10.84, P < 0.001). Male mortality was more rapid than that of females.

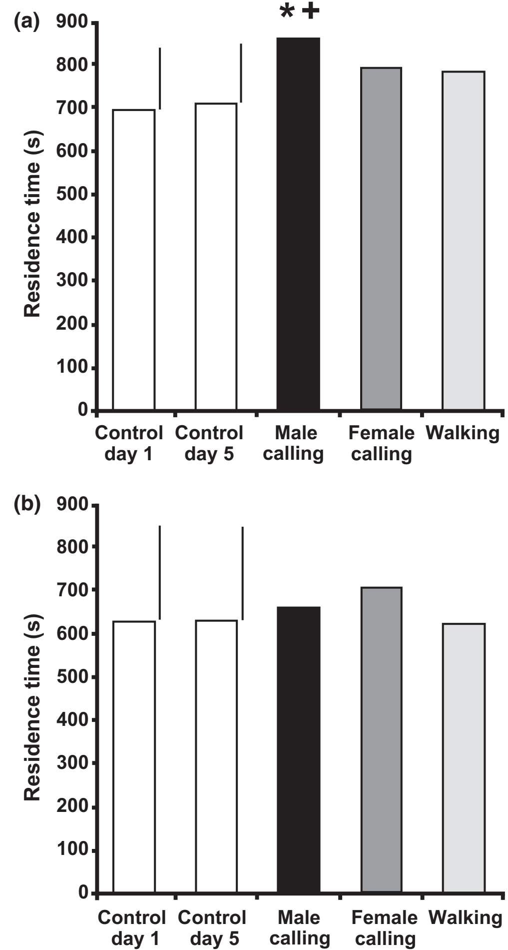

Fig. 5 The mean time that *Enoplognatha ovata* (a) and *Pardosa amentata* (b) spent on a plant in the presence and absence of playback stimuli. A priori application of least significant differences, following two-way ANOVA comparing playback treatments with the no stimulus condition (controls on day 1 and day 5), showed that the male calling signal significantly increased residence time of *E. ovata* on the plant (* and + indicate significant differences P < 0.05 with controls on days 1 and 5, respectively). LSDs (vertical bars) were 143.4 (ANOVA incorporating control day 1) and 138.2 (control day 5). Leafhopper vibrational signals had no effect on residence time of *P. amentata* on the plant. LSDs were 222.6 (ANOVA incorporating control day 1) and 220.4 (control day 5).

irregular three-dimensional tangled web underneath a living leaf (Wise & Reillo 1985) and it is not clear to what extent its web is used for capturing prey (Agnarsson 2004; Eberhard *et al.* 2008). Our observations in the field and from microcosm experiments also indicate that *E. ovata* remains in direct contact with the leaf most of the time. Although the foraging behaviour of *E. ovata* has not been investigated in detail, it has been reported that it often changes its foraging site (Greco & Kevan 1999). Some other species of web-building

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spiders also forage away from their webs (Uetz *et al.* 1999), and this may also be the case for *E. ovata*.

Sensory cues from prey play an important role in site-selection in arthropod predators (Persons & Uetz 1996; Scharf & Ovadia 2006), and vibrational signals from the more mobile males may be more easily exploited by predators as information relevant to choosing foraging sites, rather than more precise localization cues. Assessing patch quality (i.e. food availability) before selecting foraging sites is particularly important for less mobile predators (Uetz 1992) and may be especially valuable in a complex environment in which prey distribution varies unpredictably in time and space (Persons & Uetz 1996). While our results, both molecular and behavioural, contradict other studies regarding the importance of wolf spiders as predators of leaf- and planthoppers, they are consistent with previous work showing that these spiders do not use incidental vibrational cues alone to assess site quality (Persons & Uetz 1996).

Trophic ecology is not simply a matter of relative population densities and encounter rates, but is often the product of a suite of complex and highly evolved behavioural mechanisms. Although we did not address the mechanism(s) underlying selective spider response to male vibrational signals, there are several possible explanations. Because in *A. makarovi* male vibrational calls always have higher amplitude than female replies and incidental vibrational cues induced by walking leafhoppers (see Fig. S1, Supporting information, M. Virant-Doberlet, unpublished data), spider behaviour may be influenced by signal amplitude. However, in our experiments, we did not reverse the amplitude relationship between different types of stimuli, and therefore, further studies are needed to resolve this question. Another possibility is that spiders might also be able to use more complex temporal and spectral characteristics to distinguish between vibrational signals. Recently, it has been shown that wolf spiders perceive bird calls as substrate vibrations and they can use these signals to distinguish between threatening and non-threatening species (Lohrey *et al.* 2009). The most intriguing possibility, however, is that *E. ovata* learned to associate these vibrational signals with food availability. Learning (behavioural change caused by experience) can be a key to understanding trophic interactions (Kondoh 2010). Cognitive abilities of spiders are well established (Wilcox & Jackson 1998; VanderSal & Hebets 2007), and experience plays an important role in site-selection (Morse 2000) and prey preference (Punzo 2002; Jackson & Li 2004). Furthermore, it has been shown recently that predatory pit-building antlions (sedentary insect larvae; Neuroptera: Myrmeleontidae) can associate a behaviourally neutral vibrational cue with the arrival of the prey and that such learning increases fitness by improving their digestive efficiency (Guillette *et al.* 2009). Because spiders used in playback experiments were collected in the field, it is possible that at least some of them would have encountered *A. makarovi* vibrational signals in their environment and learned to associate them with prey. It has been shown that in jumping spiders, a single encounter is sufficient for forming selective attention to specific features of a prey (Jackson & Li 2004), while antlions learned to associate a vibrational cue with food after only two training sessions (Guillette *et al.* 2009). The fact that some spiders used in the playback experiments might associate vibrational signals of *A. makarovi* males with prey, because of their previous experience in the field, could also explain variability in spiders' responses. While some spiders showed no response at all, others oriented towards and located the source of vibrations. It is interesting to note that the aggressive mimicry repertoire of the araneophagic jumping spider *Portia fimbriata* also includes mimicry of the male vibrational courtship display of another locally abundant jumping spider (Jackson & Wilcox 1990; Jackson & Pollard 1996). The facts that experience and associative learning play an important role in spider foraging decisions (Morse 2000; Punzo 2002; Jackson & Li 2004), and that predation behaviour can show geographic variation because of different selection pressures (Jackson & Carter 2001), are likely to increase experimental error rates. The fact that we obtained significant results, despite such high variance, strongly supports the case for exploitation by predators of species- and sex-specific signals.

Although we showed that spiders can exploit sex-specific insect vibrational signals, we did not directly address the question of the cost of this sexual trait (Andersson 1994; Kotiaho *et al.* 1998; Kotiaho 2001). Our results indicate that, contrary to widespread belief, the evolution and design of vibrational signals and signalling behaviour are likely to be affected by a trade-off between natural and sexual selection, because of the direct cost imposed by vulnerability to predation (Burk 1982; Andersson 1994; Kotiaho *et al.* 1998; Zuk & Kolluru 1998; Kotiaho 2001). Despite the fact that vibrational signalling is a widespread form of communication, it is still the least understood channel of communication (Virant-Doberlet & Čokl 2004; Cocroft & Rodríguez 2005; Hill 2008, 2009). In particular, very little is known about ecological sources of selection on vibrational communication, especially the interactions between vibrational signallers and their predators. Many interesting behavioural, ecological and evolutionary questions remain unanswered, which should be addressed in further work. Sexual and natural selection may be interacting to maximize efficiency of sexual

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communication while minimizing exposure to interception and exploitation by predators. Male leafhoppers may be easier to detect than females, because they advertise themselves over a longer period with structurally more complex and conspicuous signals of high amplitude (Burk 1982; Zuk & Kolluru 1998; Haynes & Yeargan 1999). However, they may also be harder to localize, because males frequently change their position, either during initial advertising or while searching for a replying female. The replies from stationary females might, therefore, be easier to locate. Potentially, female vibrational signals may also be easier to interpret by unintended receivers, because they are, in general, less complex and follow a similar design between species (simple trains of regularly repeated pulses) (Čokl & Virant-Doberlet 2003). We need to ask how different vibrational signals from different species of leafhopper might be exploited by predators, whether they learn, for example, the signals of locally abundant prey species or are responding to signal qualities that are common across many prey species. Interception of vibrational communication may well be a widespread mechanism for prey location, one that is likely to be a major driver of evolution in both predators and prey. We recommend the combination of molecular analysis of predation in the field, with behavioural work in the laboratory, as a blueprint for future work in this newly revealed area of trophic ecology.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Target and non-target species used in cross-amplification tests with the *Aphrodes* primers in Table 1.

Fig. S1 Oscillograms of representative *Aphrodes makarovi* vibrational signals. (A) male calling signals, (B) female calling signal, (C) incidental vibrational cues induced by walking leafhoppers.

Fig. S2 Size difference between *Aphrodes* leafhoppers and *Pardosa* and *Enoplognatha* spiders used in microcosm experiments. Mean values together with minimum and maximum measured values are shown.

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