RESEARCH ARTICLE

Developing a paired-target apparatus for quantitative testing of nest defense behavior by vespine wasps in response to con- or heterospecific nest defense pheromones

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Abstract

Social wasps commonly exhibit impressive, pheromone-mediated nest defenses with stinging attacks on potential vertebrate nest predators. Studying this type of nest defense and comparing results across studies is challenging because there is no standardized method for quantifying defense intensities. For that reason, we developed a simple, paired-target apparatus coupled with easy and inexpensive data recording and analysis technologies. Each target is formed by two conjoined black plastic weigh boats that generate distinct percussive sounds when struck by attacking wasps. A battery-powered microphone inside each target converts the sounds into electrical signals that are transferred to a digital audio recorder. These audio files are then split into left- and right-channel files, saved as 16-bit WAV files, and the strikes to each target are counted using the open-source software SoundRuler. Using this apparatus, we show that workers of *Vespula pensylvanica*, *V. alascensis*, and *V. germanica* strike targets that are treated with conspecific venom sac extract more frequently than paired control targets. We also show that workers of *V. alascensis*, *V. pensylvanica* and *V. germanica* strike targets that are treated with heterospecific extracts more frequently than paired control targets, indicating that the wasps recognize nest alarm pheromones from congeners. These data provide evidence for conserved nest defense pheromones among some *Vespula* wasps and proof of concept that our technology is capable of quantifying the intensity of pheromone-mediated nest defense behavior in *Vespula* and other large and formidable social wasps.

Introduction

Nest defense is an integral life history trait of social insects. Many social wasps and bees are capable of coordinated, massed stinging attacks against potential vertebrate nest predators. During nest defense, large numbers of workers are mobilized and engage in stinging, biting or venom spraying to dissuade potential nest predators (Smith et al. 2001). These defense tactics are trademarks of many vespine wasps and have earned them a fearsome reputation.

Nest defense is often coordinated by alarm and marker pheromones released by worker wasps. The alarm pheromone recruits nest mates out of the nest, and the marker pheromone that is deposited on potential predators directs the attacking workers toward them (Verheggen et al. 2010). In social insects with autotomizing stings, the (barbed) stinger and its associated venom sac becomes severed from the stinging insect and then remain attached to the host. If the venom sac contains marker pheromone, the stung predator is effectively marked for attack (Hermann 1971, Overal et al. 1981, Mulfinger et al. 1992). In other social species, such as hornets (*Vespa*) and some yellowjackets (*Vespula*), alarm and marker pheromones are present in the venom sac, and may be deposited on a predator's skin during stinging or venom-spraying (Veith et al. 1984, Landolt et al. 1995).

Pheromone-mediated nest defense seems to be widespread in vespines (Bruschini et al. 2010). Several vespines reportedly orient toward nest defense pheromones extracted from the venom sac of conspecific workers, and attack targets treated with such extract at a greater rate than similar untreated targets (Landolt et al. 1995). With southern yellowjackets, *V. squamosa* (Drury), and eastern yellowjackets, *V. maculifrons* (Buysson, 1905), sharing at least one alarm pheromone component [3-methyl butylacetamide (Heath and Landolt 1988, Landolt et al. 1995)], it is conceivable that *V. squamosa* and *V. maculifrons* recognize and respond to each other's alarm pheromones. The ability to recognize heterospecific nest defense pheromones may be adaptive in that worker wasps may recognize the approach of a wasp nest predator marked with heterospecific alarm pheromone and then quickly stage a defense even before the nest has come under attack.

Studying nest defense of vespines is destined to reveal complex and intricate communication systems, but these studies are challenging in that nest defense is difficult to quantify. Moreover, results of previous studies are difficult to compare because they were obtained using rather different recording technologies and experimental designs. Our objectives were (1) to design an apparatus for quantifying nest defense behavior, and (2) to test experimentally whether *Vespula* workers respond to nest defense pheromones from both con- and heterospecifics.

Materials and methods

Experimental insects

We worked with western yellowjackets, *Vespula pensylvanica* (de Saussure), common yellowjackets, *V. alascensis* Packard, and German yellowjackets, *V. germanica* (Fabricius) which are the most prevalent of the ground nesting vespines in suburban British Columbia (BC). All three species nest either underground or in cavities (Boieeie 1983). Workers hunt small prey and scavenge for carrion to feed larval brood in their nests. The habit of scavenging makes workers very evident in late summer, when they enter yards and dwellings in search of food.

The experimental nests we studied in behavioral tests were located near Vancouver in the municipalities of Langley, Burnaby, and Richmond. We sourced workers for pheromone extraction at separate nests in these municipalities.

Extraction of venom sacs

We captured worker wasps emerging from their nests by placing a 4-litre glass jar with a steel-mesh cone over the nest entrance. We immediately killed and froze captured wasps by crumbling powdered dry ice into the capture jar, emptied the frozen wasps into polyethylene bags in an icebox, and transported them back to the laboratory for dissection. We excised their venom sacs, placed them in acetonitrile, macerated the tissue with a clean metal rod, and filtered the extract through glass wool to remove tissue fragments. We kept extracts frozen at a concentration of one venom sac per 10 µl, and transported extracts in an ice chest to the field for testing.

Design of the paired-target apparatus

The design of our paired-target apparatus and its recording technology was inspired by Visscher and Vetter (1995) who engineered a device for counting strikes against a target using microphones as transducers. Unlike Visscher and Vetter (1995), we did not use an electronic counter but instead recorded audio files and analyzed them later with automated counting software. We designed our apparatus so that it (*i*) required only inexpensive parts for assembly, (*ii*) recorded two channels, and (*iii*) allowed data analyses with open-source software. The paired design was inspired by Visscher and Vetter (1995), who built a paired apparatus, but never used it, and suggested it for alarm pheromone studies.

The paired-target, tripod-mounted apparatus consists of a crossbar supporting two targets separated by 1 m (Fig. 1a). The crossbar bearing the microphones is mounted to the quick-release plate of the tripod which is placed near the entrance of a *Vespula* nest
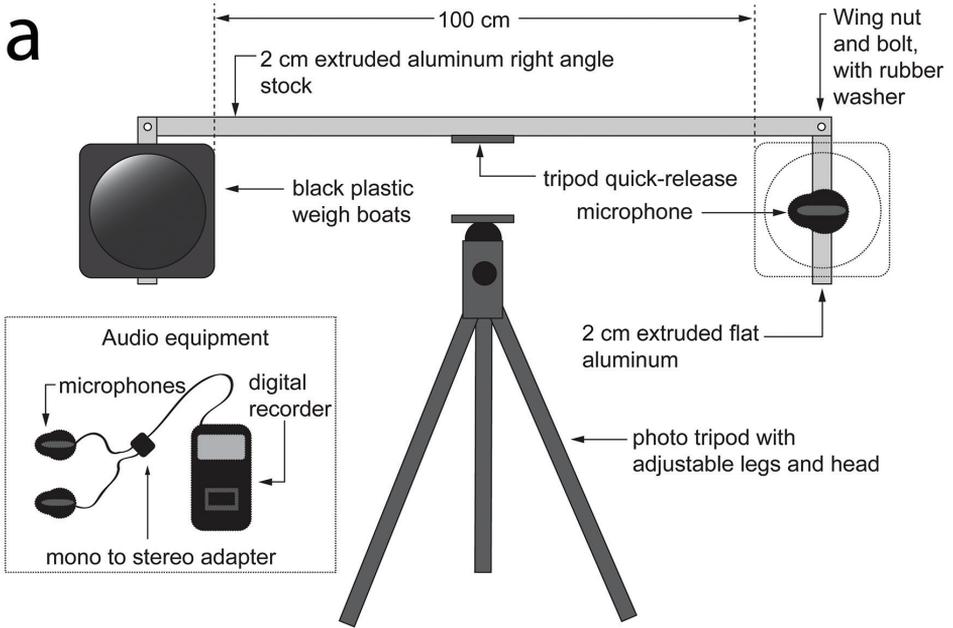
Figure 1. Graphical illustration (**a**) and photograph (**b**) of the paired-target apparatus and the recording technology deployed to bioassay pheromone-mediated nest defense in *Vespula* wasps. In b, the apparatus is placed near the entrance of a subterranean vespine nest.

(Fig. 1b), with the two targets equidistant to the nest entrance. Each of the two targets is formed by two black plastic weigh boats (13.97 cm²; Big Science Inc., Huntersville, NC, USA) that are conjoined with adhesive tape to act as a percussive medium, generating distinct sounds when struck by attacking wasps. Each target houses a Sony ECM-T6 tie-clip microphone (Sony Corporation, NY, NY, USA) powered by lithium watch cells (Fig. 1). Each of the two microphones is connected to a stereo digital audio recorder (Edirol R-09 HR, Roland Canada Ltd., Richmond, BC, Canada), with the microphone leads secured by tape along the crossbar, and fed into a two-mono-to-one stereo adapter plug. A stereo extension cable with a 3.5-mm jack extends the stereo signal cable to a convenient and safe distance from the nest for monitoring the recording event. Higher-quality shielded cables may be necessary depending on the length of the cable run and the ambient RF environment. For recording the signal from the microphones, we used an Edirol R-09 HC (Roland Corporation, Richmond BC) stereo field recorder. Other recorders may be used, but it is crucial that the two channels not be mixed in the final stereo signal.

General experimental design for testing nest defense

We studied nest defense behaviour with nests of *Vespula pensylvanica* in August 2010, and with nests of *V. alascensis* and *V. germanica* in August and September of 2011 and 2012. Invariably, we wore bee suits and veils, and retreated after disturbing a nest. We placed the paired-target apparatus 1 m from a nest entrance and recorded the wasps' responses for 1 min. We then disturbed the nest by tapping the nest entrance three times with a stick and recorded for 9 min. We repeated the test with new plastic targets, alternating the left-right position of treatment and control targets (see Supplemental Video 1).

Specific experiments

Exp. 1: Effect of target color on wasp responses

To assess the suitability of our paired-target apparatus and its recording technology for quantifying nest defense responses by vespines, we repeated the experiment by Visscher and Vetter (1995), using target color as the test variable, and a paired design. We worked with nests of *Vespula pensylvanica* following the general bioassay design, alternating the position of a white target and a black target on the paired-target apparatus.

Expts. 2–10: Effect of nest defense pheromones on wasp responses

We ran nest defense pheromone experiments the same way as the color experiment, except that (*i*) both targets of the apparatus were black, and (*ii*) and one target was treated with venom sac extract, hereafter "VSE" (at 5 venom sac equivalents), the other

with an equivalent amount of acetonitrile (50 µl) . Rather than randomly assigning the treatment and control stimulus to the left or right target in each test, we alternated their position between replicates, thus avoiding the possibility of a side bias.

In experiments 2, 5, 8 and 9, we tested the response of nest mates to VSE of conspecific workers (Exp. 2: *Vespula pensylvanica*; Exp. 5: *V. alascensis*; Expts. 8, 9: *V. germanica*) (Table 1), predicting that the target treated with VSE would receive a greater number of strikes by wasps than the solvent control target. In experiments 3, 4, 6, 7 and 10, we tested the response of nest mates to VSE of heterospecific workers. Specifically, we tested (*i*) responses of *V. pensylvanica* nests to VSE of *V. alascensis* workers (Exp. 3) and to VSE of *V. germanica* workers (Exp. 4), (*ii*) responses of *V. alascensis* nests to VSE of *V. pensylvanica* workers (Exp. 6) and to VSE of *V. germanica* workers (Exp. 7), and (*iii*) responses of *V. germanica* nests to VSE of *V. pensylvanica* workers.

Audio file processing and counting

For each replicate, we used Audacity (Audacity Team) to split the audio file into a left and right channel, and saved them as mono 16-bit WAV files under appropriate filenames. We then opened each file in SoundRuler (Gridi-Papp 2007), a free and open-source audio analysis software. Opening only the first 4 min of each recording, we analyzed the strikes against the target that show as sharp pulses and that correspond to percussive sounds as wasps struck the target (Fig. 2). We counted percussions using the "call recognition" ability and "auto" function of SoundRuler, and saving results as CSV text files. Filtering sounds above 900 Hz with the software's bandpass filter, we improved the signal-to-noise ratio of percussive strikes. We set strike recognition parameters as follows: amplitude peak: 1.1 ± 0.7 Pa; duration: 50 ± 0.4 ms; and interval: 500 ± 0.4 ms (see Appendix). Our settings file is available for download as Supplemental File 1 (see Data Resources). For all experiments except 9 and 10, we set the Soundruler to amplify the source file by 300%. For data analyses of experiments 9 and 10 which we ran in a noisy suburban construction setting, we set SoundRuler to amplify the source file by only 100%, thus avoiding automated counts of extraneous noise. As each file in any experimental series was assigned a unique name, it could be analyzed in one session of SoundRuler, parsing detailed results later using spreadsheet software.

Statistical analyses

Because our protocol produced paired data, we compared proportions of strikes on treatment and control targets in each replicate. In initial tests, we found a high variation in the total number of strikes on treatment and control targets between replicates, but the proportions of strikes on treatment targets was almost invariably higher, so a treatment effect became evident as a higher proportion of strikes on the treatment target than on the control target. We used a Wilcoxon Signed Rank test to determine whether the proportion of strikes on the treatment target differed from 0.5, and we
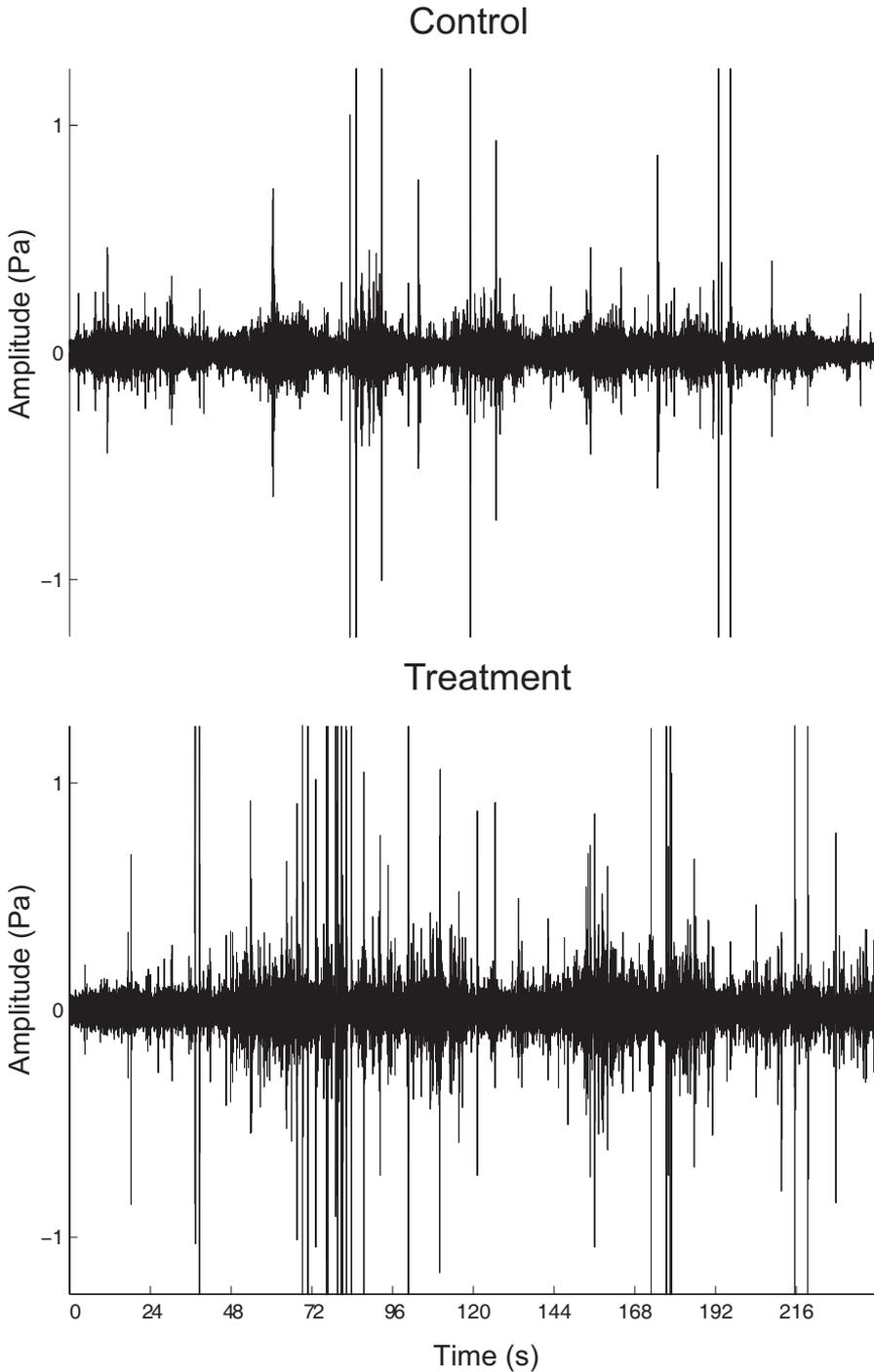
Figure 2. Representative example of paired oscillograms (obtained during replicate 6 of Exp. 2), depicting strikes caused by wasps hitting the control and treatment target of the bioassay apparatus (Fig. 1). Venom gland extract of worker wasps and acetonitrile at equivalent amount as a solvent control were applied to the treatment and control target, respectively (see methods for details).

also report the results of parametric paired T-tests for the same data. The more conservative Wilcoxon test has lower power because it uses ranks and discards ties, however, in all but one experiment the results agreed with those of the parametric tests.

Data resources

The audio data underpinning the analyses reported in this paper, as well as supplemental videos, figures and a SoundRuler settings file, are deposited at http://figshare.com at http://dx.doi.org/10.6084/m9.figshare.1581525

Results

Exp. 1: Effect of target color on wasp responses

Worker wasps of *Vespula pensylvanica* nests disproportionately struck black targets more often than white targets (Fig. 3, Table 1). The mean (± SE) number of strikes on black and white targets was 16.2 (± 12.75) and 0.1 (± 0.31), respectively, revealing a significant effect of target color on the wasps' responses.

Exps. 2–10: Effect of nest defense pheromones on wasp responses

Worker wasps of *Vespula pensylvanica*, *V. alascensis* and *V. germanica* nests struck targets treated with VSE of conspecific workers at a greater rate than control targets (Fig. 3, Table 1; Exp. 2: *P*>0.05; Exps. 5, 8, 9: *P*<0.05), indicating recognition of nest defense pheromone on treatment targets. We attribute the lack of statistical significance in experiment 2 to a lower than usual number of replicates (9 instead of 10 or 12; Table 1).

There was also recognition of nest defense pheromones from heterospecifics, as evident by nest mates striking targets treated with VSE of heterospecific workers at a greater rate than control targets. We demonstrated this phenomenon for (*i*) workers of *Vespula pensylvanica* nests responding to VSE of *V. alascensis* or *V. germanica* (Fig. 3, Table 1; Expts. 3, 4), (*ii*) workers of *V. alascensis* nests responding VSE of *V. pensylvanica* or *V. germanica* (Fig. 3, Table 1; Expts. 6, 7), and (*iii*) workers of *V. germanica* nests responding to VSE of *V. pensylvanica* workers (Fig. 3, Table 1; Expt. 10).

Discussion

Our experimental data coupled with personal observations in field experiments indicate that the paired-target apparatus meets all the criteria to effectively quantify nest defense behavior by vespine wasps in response to nest defense pheromones.

Table 1. The effect of color of two paired targets (Fig. 1), and of venom sac extract (VSE) or acetonitrile (CH_3CN) solvent applied to targets, on the number of strikes (mean ± SE) by *Vespula* (*V*) congeners in nest defense experiments (see text for details).

Exp.	Date	n	Color stimulus		Olfactory stimulus		Species tested	Strikes on S1	Strikes on S2	P < 0.05	
			S1	S2	S1	S2				t-test	WSR[3]
1	21Aug10	10	Black	White	None	None	*V. pensylvanica*	16.2 ± 12.75	0.1 ± 0.31	Y	Y
2	22Aug10	9	Black	Black	VSE[1] of *V. pensylvanica*	CH_3CN[2]	*V. pensylvanica*	70.7 ± 33.9	51.3 ± 46.4	N	N
3	7Sep11	10	Black	Black	VSE of *V. alascensis*	CH_3CN	*V. pensylvanica*	12.0 ± 11.1	3.5 ± 5.5	Y	Y
4	13Sep11	12	Black	Black	VSE of *V. germanica*	CH_3CN	*V. pensylvanica*	28.8 ± 20.5	10.2 ± 10.6	Y	Y
5	20Sep11	12	Black	Black	VSE of *V. alascensis*	CH_3CN	*V. alascensis*	122.1 ± 133.3	43.3 ± 120.8	Y	Y
6	12Sep11	10	Black	Black	VSE of *V. pensylvanica*	CH_3CN	*V. alascensis*	143.6 ± 152.6	18.0 ± 18.1	Y	Y
7	12Sep11	10	Black	Black	VSE of *V. germanica*	CH_3CN	*V. alascensis*	46.1 ± 48.2	6.6 ± 9.9	Y	Y
8	12Sep11	12	Black	Black	VSE of *V. germanica*	CH_3CN	*V. germanica*	24.8 ± 26.1	8.0 ± 11.2	Y	N
9	13Sep12	10	Black	Black	VSE of *V. germanica*	CH_3CN	*V. germanica*	6.1 ± 3.2	1.4 ± 1.1	Y	Y
10	9Sep11	12	Black	Black	VSE of *V. pensylvanica*	CH_3CN	*V. germanica*	31.2 ± 23.0	13.8 ± 9.5	Y	Y

[1] Venom sac extract (VSE) of 5 worker wasps in 50 µl of acetonitrile (CH_3CN)

[2] 50 µl of acetonitrile (CH_3CN)

[3] Wilcoxon Signed Rank test
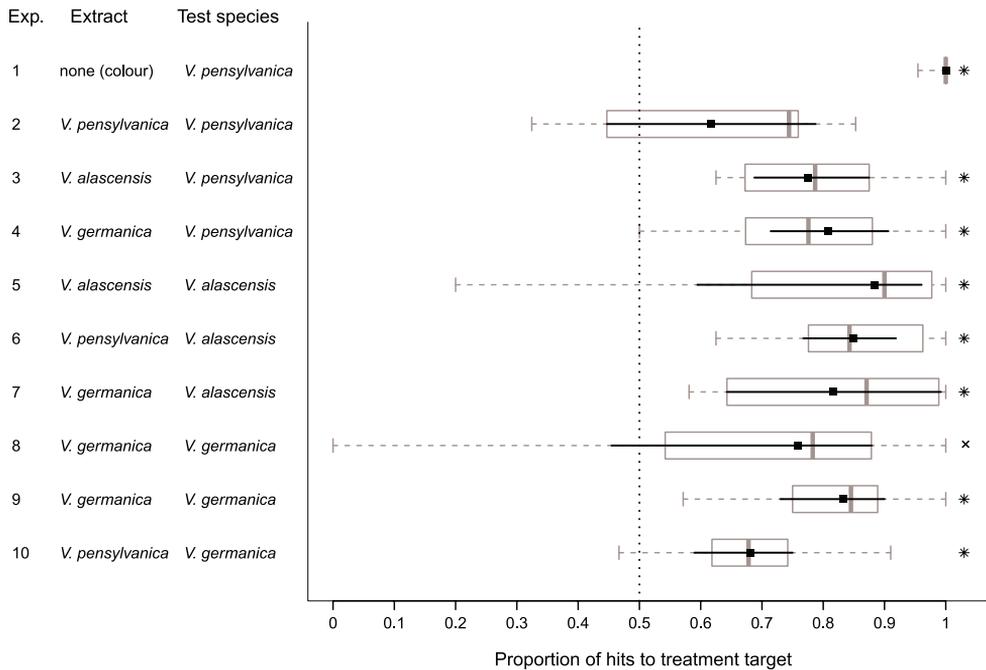
Figure 3. Proportion of strikes by various *Vespula* wasps on white or black targets in color discrimination experiment 1, and on pheromone-treated or control targets in experiments 2-10. In all experiments except 1, the control target was treated with acetonitrile. Gray boxplots show the medians (vertical lines), interquartile ranges (boxes), and ranges (whiskers). Pseudomedians and 95% confidence intervals are shown in black. Asterisks indicate pseudomedians and means that are significantly different from 0.5 using Wilcoxon Signed Rank tests and t-tests, respectively. The X in experiment 8 indicates that only the mean number of strikes on the treated target in was significantly different from 0.5.

The apparatus is assembled from inexpensive parts, its light weight facilitates transport to and from test sites, and the tripod-mount with height adjustment of the paired targets allows easy placement in uneven terrain. The conjoined plastic weigh boats serving as paired targets have surprisingly good resonant properties, thus facilitating recordings of the percussive sounds when they are struck by attacking wasps, with each strike becoming a quantifiable data point. The weigh boat targets are easily treated with test stimuli and can be readily replaced between replicates, thus avoiding the need to repeatedly clean the apparatus in a series of trials. The microphone and the digital audio recorder were sufficiently sensitive to record the wasps' strikes on targets, and "bandpass filtering" further improved the signal-to-noise ratio of these strikes. As a result, the number of strikes could be accurately counted by a software program (Sound Ruler), provided that the strike recognition parameters (amplitude, duration and inter-strike intervals) were finely tuned. Because the microphones also picked up sounds from a nearby construction site, it is advisable though to seek nests in quiet settings for data recording.

Automated counting of strikes has the advantage of expedient data processing, which is helpful when quantitative data are needed to decide on the composition of

test stimuli in follow-up experiments. The ability to run multiple sets of experiments in rapid succession is particularly critical in nest-defense pheromone research, where often many experiments are required to unravel the composition of complex pheromone blends (Veith et al. 1984, Dani et al. 2000, Wager and Breed 2000, Fortunato et al. 2004, Ibarra et al., unpubl.), and where research progress is tied each year to the few weeks during which vespine nests are in defense mode.

Exposing nests to paired rather than single targets provided the option to compare and analyze proportions, instead of absolute numbers, of strikes on treatment and control targets. This option proved valuable because a nest's propensity to defend in response to a test stimulus varied between days or replicates, a fact that renders the absolute number of strikes as an assessment criterion for the potency of a test stimulus more difficult to interpret.

Our data support evidence for the presence of nest defense pheromones in *Vespula pensylvanica*, *V. alascensis* and *V. germanica* (Fig. 3, Table 1, Exp. 2, 5, 8, 9). Alarm pheromone activity has previously only been noted for one of these species, *Vespula germanica* (Maschwitz 1964). Much greater rates of attack by *V. pensylvanica* workers on black targets than on white targets (Fig. 3, Table 1, Exp. 1) support similar results from a previous study (Visscher and Vetter 1995), and suggest that vespines in nest-defense mode respond to visual cues associated with potential nest predators.

Intriguingly, our data also provide evidence that vespines respond not only to their own nest defense pheromones but also to those of heterospecifics. Workers of *Vespula alascensis*, *V. germanica* and *V. pensylvanica* all struck targets treated with VSE of heterospecifics more frequently than paired control targets (Fig. 4, Table 1), indicating that they recognize nest defense pheromones from congeners. The underlying mechanisms are likely one or more pheromone components that are shared between congeners. *N*-3-methylbutylacetamide, for example, is an alarm pheromone component of both southern yellowjackets, *V. squamosa* (Drury), and eastern yellowjackets, *V. maculifrons* (Buysson). There are also common acetamides in VSEs of *V. alascencis*, *V. germanica* and *V. pensylvanica* (McCann et al., unpubl. data), one or more of which may have a nest defense pheromone function and may have contributed to the emerging evidence for conserved alarm pheromone chemistry in *Vespula* (Fig. 3, Table 1).

Each species will likely respond most vigorously to its own nest defense pheromone, because the alarm message is released by nest mates when they sense an immediate threat to the nest and when concerted defense by nest-mates is needed to protect the nest's offspring. Nonetheless, the recognition of nest defense pheromones from *Vespula* congeners seems advantageous because congeners sometimes nest in close proximity. If a nest were to be attacked by a vertebrate predator, and marked with nest defense pheromone while being stung by defending nest mates, then this "marked" predator could be sensed from a distance by worker wasps of a congener nest allowing nest mates to stage a defense well before the predator has even reached the nest and initiated an attack.

In conclusion, we have described a paired-target apparatus that facilitates the quantification of pheromone-mediated nest defense behavior by vespine wasps, and provide evidence that some *Vespula* species respond to nest defense pheromones of both con- and heterospecifics. This work provides the means and incentive to study this phenomenon more closely and to chemically identify the defense pheromones of *Vespula* species.

Acknowledgements

References

Boieeie WO (1983) Control of yellowjackets (Hymenoptera: Vespidae) in British Columbia. MPM Thesis. Simon Fraser University, Burnaby, British Columbia.

Bruschini C, Cervo R, Turillazzi S (2010) Pheromones in social wasps. In: Vitamins and Hormones. Elsevier Inc., 447–492. doi: 10.1016/S0083-6729(10)83019-5

Dani FR, Jeanne RL, Clarke SR, Jones GR, Morgan ED, Francke W, Turillazzi S (2000) Chemical characterization of the alarm pheromone in the venom of *Polybia occidentalis* and of volatiles from the venom of *P. sericea*. Physiological Entomology 25: 363–369. doi: 10.1111/j.1365-3032.2000.00205.x

Fortunato A, Dani FR, Sledge MF, Fondelli L, Turillazzi S (2004) Alarm communication in *Ropalidia* social wasps. Insectes Sociaux 51: 299–305. doi: 10.1007/s00040-004-0744-2

Gridi-Papp M (2007) SoundRuler: Acoustic Analysis for Research and Teaching. http://soundruler.sourceforge.net

Heath RR, Landolt PJ (1988) The isolation, identification and synthesis of the alarm pheromone of *Vespula squamosa* (Drury) (Hymenoptera: Vespidae) and associated behavior. Experientia 44: 82–83. doi: 10.1007/bf01960257

Hermann HH (1971) Sting autotomy, a defensive mechanism in certain social Hymenoptera. Insectes Sociaux 18: 111–120. doi: 10.1007/BF02223116

Landolt PJ, Heath RR, Reed HC, Manning K (1995) Pheromonal mediation of alarm in the eastern yellowjacket (Hymenoptera: Vespidae). The Florida Entomologist 78: 101–108. doi: 10.2307/3495673

Maschwitz UW (1964) Alarm substances and alarm behaviour in social Hymenoptera. Nature 204: 324–327. doi: 10.1038/204324a0

Mulfinger L, Yunginger J, Styer W, Guralnick M, Lintner T (1992) Sting morphology and frequency of sting autotomy among medically important vespids (Hymenoptera: Vespidae) and the honey bee (Hymenoptera: Apidae). Journal of Medical Entomology 29: 325–328. doi: 10.1093/jmedent/29.2.325

Overal W, Simoes D, Gobbi N (1981) Colony defense and sting autotomy in *Polybia rejecta* (F)(Hymenoptera: Vespidae). Revista Brasiliera de Entomologia 25: 41–47.

Smith AR, Donnell SO, Jeanne RL (2001) Correlated evolution of colony defence and social structure: A comparative analysis in eusocial wasps (Hymenoptera: Vespidae). Evolutionary Ecology Research 3: 331–344.

Veith HJ, Koeniger N, Maschwitz U (1984) 2-Methyl-3-butene-2-ol, a major component of the alarm pheromone of the hornet *Vespa crabro*. Naturwissenschaften, 328–329. doi: 10.1007/bf00396622

Verheggen FJ, Haubruge E, Mescher MC (2010) Alarm pheromones-chemical signaling in response to danger. Vitamins and hormones 83: 215–39. doi: 10.1016/S0083-6729(10)83009-2

Visscher PK, Vetter RS (1995) Smoke and target color effects on defensive behavior in yellowjacket wasps and bumble bees (Hymenoptera: Vespidae, Apidae) with a description of an electronic attack monitor. Journal of Economic Entomology 88: 579–583. doi: 10.1093/jee/88.3.579

Wager B, Breed M (2000) Does honey bee sting alarm pheromone give orientation information to defensive bees? Annals of the Entomological Society of America 93: 1329–1332. doi: 10.1603/0013-8746(2000)093[1329:DHBSAP]2.0.CO;2

Apppendix
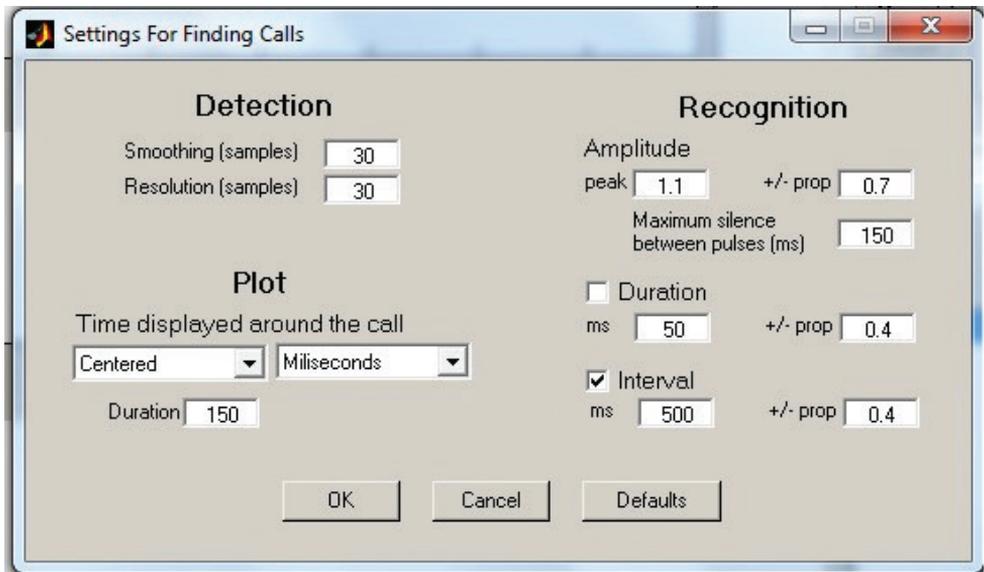
Call recognition parameters used in SoundRuler software for detecting and counting wasp strikes against plastic targets. In addition to these settings, we also set a bandpass filter of 900 Hz.