

## Behavioral and olfactory responses of grasshopper hatchlings, *Melanoplus sanguinipes*, to plant odours and volatile compounds

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**Abstract** Behavior and olfactory responses of grasshopper hatchlings, *Melanoplus sanguinipes* (F.), to odours from plant foliage and volatile compounds were tested using a glass Y-tube olfactometer and electroantennogram (EAG) techniques respectively. In single choice trials, newly hatched hoppers were much more sensitive to the odour from intact leaves and chopped foliage of ryegrass and wheat than other plants. Chopped sorghum leaves, but not stem-cut sorghum, were also significantly attractive. The orientation responses of grasshopper hatchlings to these plants were highly consistent with those of last instar hoppers and adults. When ryegrass was employed as the control, the odour from stem-cut alfalfa was more attractive. There was no significant difference in hopper orientation responses to the odours from chopped seedlings of sorghum, alfalfa, wheat or ryegrass. However, significantly more hoppers preferred the chopped ryegrass control to chopped Louisiana sage. Measurement of the EAG response of first instar hoppers to these plant odours showed that the odour of Louisiana sage elicited the greatest response amplitudes. In olfactory tests using different volatile components, Z-3-hexenol, E-3-hexenol, Z-hex-3-enyl acetate, E-2-hexenal and hexenal gave greater EAG responses than geraniol and 1-octen-3-ol. These results are also consistent with comparable data from adults. Newly hatched grasshoppers had similar EAG response profiles to plant materials and chemicals to those of adults, although the absolute EAG values of young hoppers were much lower than those of adults. Therefore, newly hatched hoppers were able to distinguish plants from an air control, and even host plants from non-host plants, and the feeding experience of hoppers probably has little influence on their subsequent ability as adults to identify and locate food plants.

**Keywords:** grasshopper, *Melanoplus sanguinipes*, hatchling grasshoppers, odour, olfaction, EAG, host plant location behaviour.

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Host-plant location and recognition in herbivorous insects is composed of an ordered set of behavioral responses to stimuli from host and non-host plants<sup>[1]</sup>.

Odours associated with host plants provide cues to the availability of food, oviposition sites, and other information beneficial to survival and reproduction<sup>[2]</sup>. A lot of studies have shown that grasshoppers, locusts and crickets are able to orientate to plant leaves by tracking the odour of volatile components and blends of these substances<sup>[3–11]</sup>. Hopkins & Young<sup>[10]</sup> and Szentesi et al.<sup>[11]</sup> stated that some volatile compounds could play an important role in the relatively long-distance location of host plants by grasshoppers. Once these insects are attracted by potential host plants, *Melanoplus* spp. performs a series of exploratory and gustatory behaviors<sup>[12]</sup>. Blaney<sup>[13]</sup> reported that the differential components and thickness of leaf waxes from host and non-host plants affect the ability of locusts to differentiate these plants. Therefore, the initiation of the first steps in the feeding behavior of grasshoppers could be dependant on the detection of specific odours from food plants<sup>[10,11]</sup>. This leads us to assume that these odours are crucial cues that enable the newly hatched hoppers to rapidly find suitable host plants after hatching.

*Melanoplus sanguinipes* (Orthoptera: Acrididae) is a polyphagous grasshopper species, which varies in its relative utilization of grasses and dicots and overall diet breadth<sup>[12]</sup>. There are significant differences between olfactory and gustatory responses of specialist and generalist grasshoppers to potential food plants<sup>[12,14]</sup>. The age and feeding history of insects largely depends on sensory variability<sup>[15–18]</sup>. Polyphagous grasshoppers are known to be able to learn to avoid foods that were associated with an artificial aversive stimulus<sup>[19,20]</sup>. Some studies have also found that the olfactory sensitivity and food preferences of insects change according to feeding experience and exposure to special chemicals<sup>[21–24]</sup>.

In most cases, electrophysiological studies of the host odour response in grasshoppers have been too narrow in scope<sup>[25]</sup>. In fact, little is known about the role of feeding experience, learning and memory in insect chemoreception. Previous study has shown that the fifth instar hoppers of *Schistocera gregaria* could be induced to walk upwind in the laminar airflow of a wind tunnel when crushed grass was placed in the upwind section<sup>[3]</sup>. However, the behavioral and electrophysiological responses of early instar hoppers to plants and volatile chemicals have not been well studied.

Although the most distal annuli of hoppers bear their full complement of sensilla at hatching<sup>[26]</sup>, little attention has been paid to their role in host plant orientation and olfaction. Few studies have examined difference in the responses of young and adult hoppers to plant volatiles. Newly hatched hoppers, lacking prior feeding and chemoreception experience, are a kind of ideal model insect for such research. This paper describes the results of behavioral and electrophysiological experiments designed to determine the ability of newly hatched first instars of a polyphagous grasshopper to discriminate between differ-

ent types of plant chemical information. The general aim was to determine some of the behavioral and olfactory cues that elicit host orientation and selection in newly hatched grasshoppers, and more specifically to address the following questions: (i) Are newly hatched grasshoppers able to distinguish between host and non-host plants in an olfactometer? (ii) Can they successfully select suitable host plants? (iii) Are there different behavioral and olfactory responses to intact vs. chopped plants? (iv) Are there differences between grasshopper hatchlings and adults in plant orientation and electroantennogram responses?

## 1 Materials and methods

(i) Experimental insect. Eggs of the grasshopper, *Melanoplus sanguinipes*, were obtained from a non-diapausing laboratory colony at the USDA Rangeland Insect Laboratory, Bozeman, Montana. Newly hatched hoppers were held without any food but supplied with water for 8–10 h prior to testing. About 30 min before testing individual hoppers were placed in glass tubes with nylon netting covering the openings. A group of 10 insects were used for each test with 3–7 replicates for each treatment. All test insects were used only once.

(ii) Olfactometer apparatus. A Y-tube olfactometer<sup>[10]</sup> was used to examine the attraction of grasshopper hatchlings to plant foliage and volatile compounds. The olfactometer and test methods used here were the same as described by Hopkins and Young<sup>[10]</sup>. Compressed air was humidified by being passed through distilled water in a gas-washing bottle, and then split into two columns using a flowmeter equipped with needle valves. The flow rate of air to both arms of the Y-tube was regulated at 375 mL/min. The air stream on the treatment side was passed through a flask containing the odour source before entering one arm of the Y-tube. Hoppers were observed for 15 min and the numbers of insects in each arm of the Y-tube were recorded. The attractiveness of each odour was expressed as the mean percentage of hoppers that entered the treatment arm. Hoppers that remained inactive were considered to have not made a choice and were not included in this analysis.

(iii) Plant foliage trials. The attractiveness of five host plants species, ryegrass (*Lolium perenne*), wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*) and alfalfa (*Medicago sativa*), and one non-host plant Louisiana sage (*Artemisia ludoviciana* Nutt.)<sup>[7,13]</sup>, were tested with the Y-tube olfactometer. With the exception of Louisiana sage, which was collected in the field, all stem-cut and chopped plant samples were grown from 5–7 d old seedlings in a greenhouse. The sage was collected in plastic bags, which were then sealed and immediately transported to the laboratory where they were stored in a refrigerator. All plant materials were kept at room temperature for 1 h prior to testing. Ryegrass is commonly used to rear grass-

hoppers in laboratories. Since bran is a common supplementary diet for laboratory-reared grasshoppers, dry and moist bran were presented to provide a comparison to the stem-cut plant material.

Stem-cut seedling samples (8 g) and chopped samples (4 g) were placed in two-necked flasks fitted with inlet and outlet tubes for the sample air stream, while an identical flask containing a moist cotton ball was used for the control air stream. Detailed methods and procedures are as described by Hopkins and Young<sup>[10]</sup>. A moist air stream was used as the control for single choice tests while ryegrass was the control in binary choice tests.

Percent attractiveness was calculated as the number of the hoppers entering the sample arm/total number responding to sample and control  $\times 100$ . Chi Square analysis was used to determine the significance of differences in the numbers of the hoppers entering the sample and control arms of the Y-tube. Chi Square contingency tests were used to determine the significance of differences between specific plant samples and the control. Comparisons between plant species were subjected to ANOVA and means were compared by LSD (SPSS 10.0).

(iv) Electroantennogram techniques. EAGs (in mV) were recorded from the excised left antennae of newly hatched hoppers. Since there is no significant difference in olfactory response between the sexes<sup>[7]</sup>, the sex of hoppers was not recorded. The distal tips of the antennae were cut off, and the antennae severed from the head. Each antenna was placed between the tips of two Pasteur pipettes containing cockroach saline<sup>[27]</sup>. Ag-AgCl wires were inserted into the saline from the other end of each pipette and connected to a glass R18 DC amplifier. The amplifier output was read on a Tektron 564B storage oscilloscope.

Since the amplitude of an EAG depends more on the weight of the source material than on the leaf area of plant samples<sup>[28]</sup>, only the fresh weight of plant samples was standardized. For each plant species, we chopped 0.5 g fresh leaves of the seedlings into pieces, and then placed them into a cartridge of 15 cm in length and 1 cm in diameter to act as the odour source<sup>[9]</sup>. Recordings were made from the antennae of 12 grasshoppers. Plant samples were numbered and tested randomly. At least 20 s were allowed between two stimuli for recovery of EAG.

An empty cartridge was presented after every five tests as a blank. The blank responses before and after each series of odour stimuli were averaged and subtracted from the EAG amplitudes measured for plant material to control for the effects of mechanical disturbance by airflow. As a chemical standard, a Pasteur pipette containing a filter paper strip impregnated with 25  $\mu\text{L}$  hexanol at a concentration of 10 ng/ $\mu\text{L}$  was used every five tests. The standard tests showed that no deterioration of the antennae occurred during the experiment, absolute EAG amplitudes (mV) after subtraction of the blank response were therefore used in data analysis.

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98%—99% pure quantities of seven volatile compounds (hexonal, Z-hex-3-enyl acetate, hexanol, Z-3-hexenol, E-2-hexen-1-ol, E-3-hexen-1-ol, 1-octen-3-ol and geraniol), which have been identified as major components of the plant species being tested<sup>[7,10]</sup>, were purchased from commercial sources (Aldrich Chemical Company, Milwaukee, Wisconsin and Sigma Chemical Co., St. Louis, MO). 25  $\mu$ L of each compound was pipetted onto a 1  $\text{cm}^2$  piece of filter paper at concentrations of 100 ng and 1000 ng in 1  $\mu$ L paraffin oil. Filter paper treated with 25  $\mu$ L of paraffin oil was used as a blank. As a standard, 25  $\mu$ L hexanol at concentration of 10 ng/ $\mu$ L was substituted every seven tests.

Data on the EAG responses to the five plant odours and seven chemicals were analyzed by ANOVA. Data that yielded significant  $P$  values ( $<0.05$ ) were subject to multiple comparisons using the LSD test at  $P<0.05$ .

### 2 Results

(i) Attractiveness of plant leaf odours. The odour from stem-cut ryegrass and wheat leaves were highly attractive to grasshopper hatchlings compared to the humidified air control (Fig. 1(a)). Although stem-cut alfalfa foliage was more attractive to hoppers than the control, there was no statistically significant difference between the number of hoppers that showed a preference and those

that did not make a choice. No significant difference was observed between stem-cut sorghum, Louisiana sage, dry bran, moist bran and the air control. In the Louisiana sage and alfalfa trials most hoppers did not make a choice. Dry and moist bran elicited the least response among the foods tested. The odour of stem-cut sorghum foliage was unattractive to the hoppers. With the exception of ryegrass, alfalfa and wheat there were no statistically significant differences in hopper preferences between the food plants and bran (Fig. 1(a)).

The odours from the chopped leaves of seedling ryegrass, wheat and sorghum were highly attractive to the young hoppers compared to the humidified air control (Fig. 1(b)). However, the odours from chopped alfalfa and Louisiana sage foliage were not significantly more attractive than the control. With the exception of sorghum, preferences for chopped leaf material were similar to those for stem-cut foliage. Furthermore, chopping sorghum and wheat resulted in a highly significant ( $P<0.05$  and  $P<0.01$ ) increase in their attractiveness, but the same was not true for alfalfa and Louisiana sage. Therefore, although chopped plant materials may release higher concentrations of odour molecules than stem-cut materials, the chopped foliage of alfalfa and Louisiana sage were no more attractive to hoppers than stem-cut plants. Except for ryegrass and Louisiana sage, there were no statistically significant

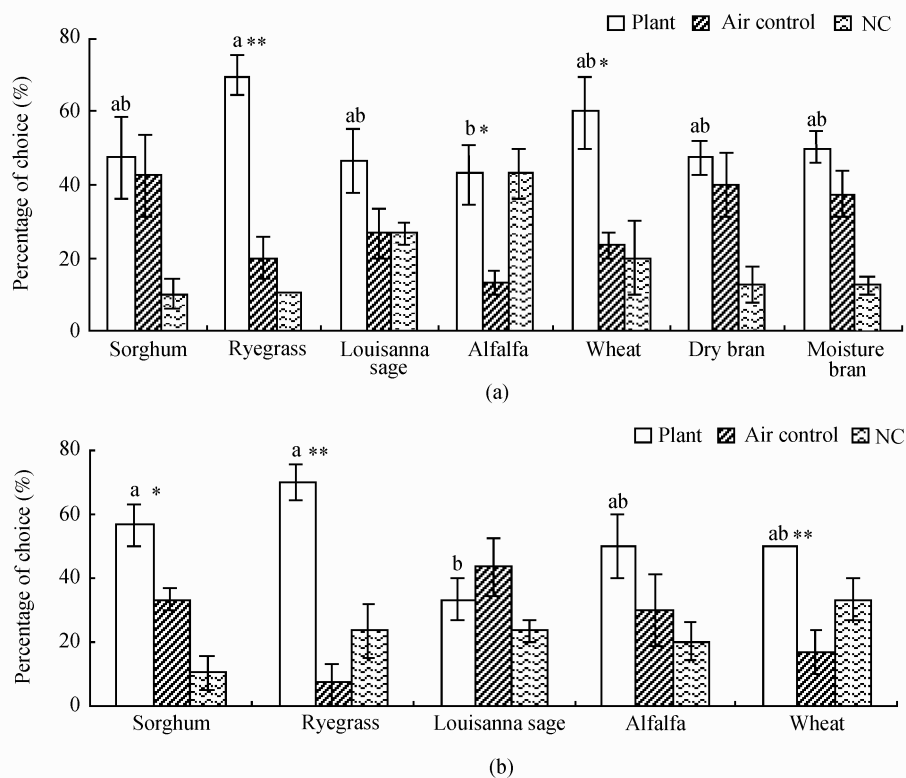


Fig. 1. Olfactory attraction of grasshopper hatchlings of *M. sanguinipes* to the odours of plant leaves versus humidified air in a Y-tube olfactometer. (a) Selection between intact leaves (stem-cut); (b) selection between chopped leaves. NC, no choice. Vertical bars indicate standard errors. Letters on the bars represent significantly different responses to different plant species at single choice trials ( $P<0.05$ ). \* Significant differences in response between the plant species and control (\*  $P<0.05$ , \*\*  $P<0.01$ ).

differences in hopper preferences between host plants (Fig. 1(b)).

(ii) Attractiveness of plant leaf odours with ryegrass as control. When chopped ryegrass was used as the control, the numbers of hoppers that made no choice were significantly reduced compared with the air control (Fig. 2). In the two-choice trials, the odour of stem-cut alfalfa was significantly more attractive (72.5%,  $P < 0.01$ ) than ryegrass (27.5%). No difference was observed between Louisiana sage and wheat foliage compared to the control. The number of hoppers preferring the ryegrass control was significantly higher ( $P < 0.05$ ) than those preferring sorghum (Fig. 2(a)). In the case of chopped foliage, the odour of sorghum, alfalfa and wheat had similar attractiveness to hoppers as the ryegrass control. However, chopped Louisiana sage was significantly less attractive compared to the same control (Fig. 2(b)).

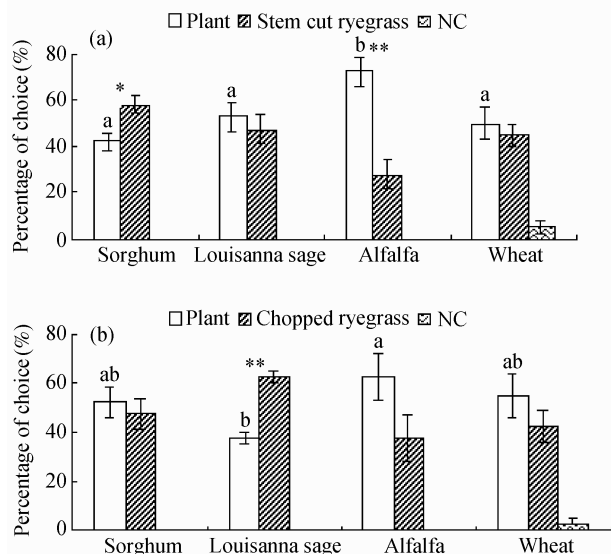


Fig. 2. Olfactory attraction of grasshopper hatchlings of *M. sanguinipes* to the odours of plant leaves versus chopped ryegrass in a Y-tube olfactometer. (a) Selection between intact leaves (stem-cut); (b) selection between chopped leaves. Vertical bars indicate standard errors. NC, no choice. Letters on the bars represent significantly different responses to different plant species in binary choice trials ( $P < 0.05$ ). \* Significant differences in response between the plant species and ryegrass control (\* $P < 0.05$ , \*\* $P < 0.01$ ).

(iii) EAG responses of grasshopper hatchlings to host plants. The EAG responses of grasshopper hatchlings to the five plant materials were shown in Fig. 3. The odour of Louisiana sage resulted in the greatest amplitude, and was significantly higher than ryegrass, wheat or sorghum. Ryegrass, alfalfa and Louisiana sage elicited EAG responses significantly greater than the blank ( $P < 0.01$ ). No significant differences were found between the three grasses and alfalfa (Fig. 3).

(iv) EAG responses of grasshopper hatchlings to volatile chemicals. One blank (a filter paper treated with

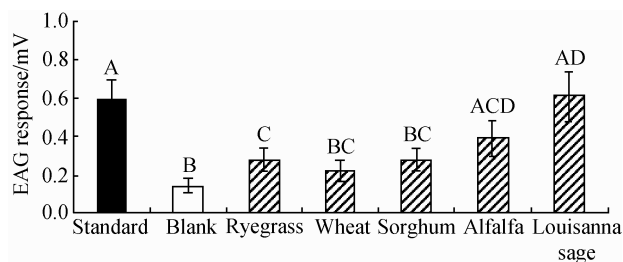


Fig. 3. EAG responses of grasshopper hatchlings of *M. sanguinipes* to odours of host plants. Vertical bars indicate standard errors ( $n=12$ ). Letters on the bars represent significantly different responses between plant species. Means with the same letter are not significantly different ( $P < 0.01$ ).

25  $\mu\text{L}$  of paraffin oil) and a standard (25  $\mu\text{L}$  hexanol at a concentration of 10  $\text{ng}/\mu\text{L}$ ) stimulus were used separately to examine the EAG responses of the hoppers to volatile chemicals. The EAG responses of the hoppers to both concentrations of the seven compounds are presented in Fig. 4. The greatest difference in EAG amplitude between the two concentrations, an increase from  $-0.35$  to  $-0.675$  mV, occurred with E-2-hexenal ( $P < 0.05$ ). However, the EAG amplitudes of the other volatile compounds did not increase significantly with the increase in concentration from 100 to 1000  $\text{ng}/\mu\text{L}$ . Although each compound, with the exception of geraniol at 100  $\text{ng}/\mu\text{L}$ , elicited distinct responses compared with the blank, there was little difference in amplitude between them. The hoppers displayed lower EAG responses to E-2-hexenal and 1-octen-3-ol at 100  $\text{ng}/\mu\text{L}$  than the other substances. At the 1000  $\text{ng}/\mu\text{L}$  concentration level, there was a significant difference in EAG response between geraniol and Z-3-hexenal and hexenal. Overall, geraniol elicited the lowest EAG response at both concentrations.

### 3 Discussion

The results show that the newly hatched first instars of the grasshopper, *M. sanguinipes*, without prior experience, can detect and orient to odours of several host plants. They also appear to distinguish between the odours of host plants in a Y-tube olfactometer. The attractiveness of host plants to newly hatched hoppers was consistent with comparable data on last instar and adult grasshoppers<sup>[10]</sup>. The percent of grasshopper hatchlings that are attracted to odours in olfactometer experiments is relatively low, 50%—75% compared to 88%—97% in last instar hoppers and adults<sup>[10]</sup>. Newly hatched grasshoppers had a similar EAG response profile to adults. Both displayed greater EAG amplitudes in response to Louisiana sage and alfalfa<sup>[8]</sup>. However, first instar hoppers had much lower EAG amplitudes than adults, which may be due to their lower number of antennal sensilla<sup>[26,29]</sup>. Both last instar hoppers and adults were highly attracted to the odours of stem-cut ryegrass and wheat but not to those of stem-cut sorghum and alfalfa. However, chopping the foliage of these plants results in a highly significant increase in their attractive-

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ness<sup>[10]</sup>. Similarly, in this study, newly hatched hoppers displayed a strong preference for ryegrass and wheat in single choice trials. When chopped leaf material was tested, chopped sorghum leaves were also significantly attractive. In binary choice tests, newly hatched hoppers could distinguish stem-cut sorghum and alfalfa from stem-cut ryegrass, but not stem-cut wheat and Louisiana sage from ryegrass. In binary choice tests using chopped plant material, the young hoppers could not discriminate between sorghum, alfalfa, wheat and ryegrass, however, they always avoided selecting chopped leaves of Louisiana sage, the only non-host plant. It is very interesting that newly hatched hoppers without feeding experience were able to distinguish between host and non-host plants, in spite of the fact that this ability is related to the concentration of molecules released by the plants. Hopkins and Young<sup>[10]</sup> suggested that the amount of odour molecules released by stem-cut sorghum or alfalfa foliage may be below the grasshopper threshold of attraction.

It is generally presumed that chopping leaves will result in both an increase and change in the volatile substances released from plant material. It is therefore surprising that, although chopped sorghum was more attractive than the stem-cut plant, no other significant difference in attractiveness was found between stem-cut plants and chopped leaves. There are two possible explanations for this: The first is that the quantity of odour is not as important in determining hopper preferences as the actual blend of volatile substances; the second is that the chopping process did not result in a significant qualitative change in the plant's volatile profile. Either way, further studies are needed to test those two hypotheses. In comparison, indi-

viduals exposed to higher concentrations of volatile compounds displayed a relative high EAG response compared to lower concentrations even though this was not statistically significant (Fig. 4).

It is intriguing to note that the EAG response of grasshopper hatchlings to the odour of Louisiana sage was much greater than for ryegrass, which implies that, as suggested by other studies, the EAG response does not accurately reflect real food preferences in some situations. A specialist grasshopper, *Hypochlora alba*, that lives and feeds almost exclusively on Louisiana sage, was highly sensitive to the odour components of the host plant in both EAG and gustatory choice tests<sup>[7,14]</sup>. However, some components of the Louisiana sage had an antifeedant effect on *M. sanguinipes*. This also further illustrates the necessity of conducting bioassays rather than relying on electrophysiological analysis alone.

Another issue naturally emerging from this study is the role of host plant odour components in regard to host selection in generalist grasshoppers. Since the 5 plant species tested are widely separated taxonomically, the data from this study obviously suggest that the ratio between certain common components of plant odours is the key factor in host orientation. Analysis of the volatile components of ryegrass odour revealed that Z-hex-3-enyl acetate and the alcohol Z-3-hexenol were the dominant compounds. E-2-hexenal was found in small amounts in ryegrass<sup>[10]</sup>. All of these compounds, along with E-3-hexen-1-ol and hexenal, are the main constituents of green leaf volatiles<sup>[10,30]</sup>. When these compounds were bioassayed in the olfactometer, all were significantly attractive to grasshoppers (~75%) compared to humidified air<sup>[10]</sup>. Geraniol, a monoterpene that commonly occurs in many plant spe-

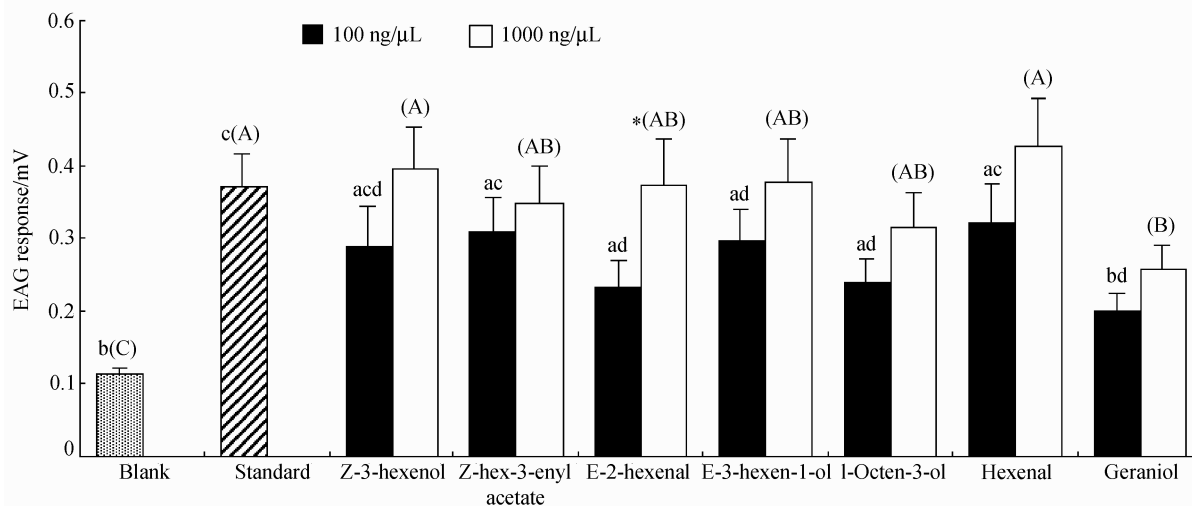


Fig. 4. Amplitudes of the EAGs recorded for grasshopper hatchlings of *M. sanguinipes* in response to volatile compounds. Vertical bars show standard errors ( $n = 12$ ). Small letters represent significantly different responses between the compounds at 100 ng/μL concentration ( $P < 0.05$ ). Capital letters in brackets represent significantly different responses between compounds at 1000 ng/μL concentration ( $P < 0.05$ ). \* Significant difference in response between the two concentrations ( $P < 0.05$ ). Blank: a filter paper treated with 25 μL paraffin oil, and standard: 25 μL at concentration of 10 ng/μL hexanol.

cies, elicited relatively weak EAG activity; the remaining compounds resulted in relatively similar EAG responses including the two predominant ryegrass volatile compounds (Z-hex-3-enyl acetate and Z-3-hexenol) that elicited the strongest response in the olfactory test. Again this substantiates the view that grasshoppers rely on the particular ratio between certain commonly occurring plant compounds in host orientation. It follows that grasshoppers recognize certain preferred host plants by their distinctive ratio of these substances, or “chemical signature”, as is the case with ryegrass.

In this study, grasshopper hatchlings displayed greater EAG responses to these compounds than other plant volatiles, such as 1-octen-3-ol and geraniol. This strongly suggests that, like last instar hoppers and adults, newly hatched hoppers also rely on detecting green plant volatile compounds to locate food plants. However, visual stimuli may also play a role in grasshopper orientation to food plants<sup>[11]</sup>. Both parents and their offspring were attracted by the odours of ryegrass, wheat and sorghum. Newly hatched grasshoppers were also able to readily detect the odour of Louisiana sage, a non-host plant but not to move towards it. Therefore, plant odour detection by this polyphagous grasshopper and subsequent discrimination of potential host from non-host plants appears to genetically fix in the newly hatched hoppers and persists during development into the adults.

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