

Advertising acceptability: is mollusk olfaction important in seedling selection?

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Abstract Although seedling herbivory is an important selective filter in many plant communities, how and why seedlings are selected is poorly understood. Here, we examined the putative role of herbivore olfaction in dictating seedling selection. Using a Y-tube olfactometer we compared snail (*Helix aspersa*) preference for pellets derived from 14-day-old macerated seedlings of nine European grassland ('Test') species against standard ('Control') pellets derived from lettuce. Snail movement towards 'Test' pellets was strongly correlated with seedling acceptability (Pearson's $r^2 = 0.86$, $P > 0.01$) and where snails exhibited a positive choice for the 'Test' species, the choice was made more quickly for highly acceptable species ($r^2 = 0.86$, $P > 0.01$). In elucidating a link between seedling acceptability and olfactory response to macerated seedlings, our study suggests that even from an early ontogenetic stage plant selection by snails may be governed by olfactory cues. This finding highlights the need for research on the role of plant volatiles in plant–herbivore interactions to consider more fully interactions operating at the seedling stage.

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Introduction

Seedling herbivory is a potent selective filter in natural ecosystems, exerting a tremendous influence over plant community composition and species coexistence (Howe et al. 2002; Hanley and Sykes 2009). However, despite its importance in plant community dynamics, the mechanisms underpinning seedling selection by herbivores are not readily apparent (Hanley and Sykes 2009). Some characteristics, such as the possession of constitutive anti-herbivore defenses strongly influence herbivore attack (Hanley and Lamont 2001), but observation of snail feeding behavior in experimental arenas suggests that some seedlings are consistently avoided even before physical contact is made (M.E. Hanley personal observation).

It is clear from studies involving established plants that olfaction is important in dictating food plant selection. Indeed, the release of volatile organic compounds (VOCs) such as terpenes and green leaf volatiles frequently results in the avoidance of the same plant by other herbivores (Laothawornkitkul et al. 2008; Unsicker et al. 2009). Fewer studies by contrast have examined whether seedlings release VOCs, although there is some evidence that invertebrates

detect seedling volatiles and modify their feeding behavior accordingly (Carroll et al. 2006, 2008).

As the principal seedling herbivore in temperate ecosystems (Crawley 1997), the olfactory response of terrestrial mollusks to plant volatiles may be particularly important. One species, *Deroceras reticulatum*, not only exhibits a strong neurophysiological response to volatile extracts from three Apiaceae species, in bioassays it also avoided consumption of standardized foods containing these same volatiles (Birkett et al. 2004). Nonetheless, while terrestrial mollusks are known to detect volatiles in established plants (Birkett et al. 2004; Unsicker et al. 2009), how VOCs influence mollusk olfaction and feeding behavior remains poorly explored, especially at the critical seedling stage where mollusks exert so much influence. The aim of this study was to investigate whether olfactory cues influence selection of seedlings by terrestrial mollusks by exposing snails (*Helix aspersa* Müller) to seedling tissue derived from grassland plant species of known acceptability.

Materials and methods

Seedling propagation and snail culture

Seeds of nine dicotyledonous grassland herb species (*Anthyllis vulneraria* L., *Medicago lupulina* L., *Ononis spinosa* L., *Plantago lanceolata* L., *Rumex acetosa* L., *R. crispus* L., *Senecio jacobaeae* L., *Trifolium pratense* L., and *T. repens* L.) and lettuce (*Lactuca sativa* L. cv. Tom Thumb) were obtained from a commercial supplier (Herbiseed Ltd, Twyford, UK). These species are commonly encountered and consumed by terrestrial molluscs, but also vary in their acceptability to both slugs and snails in field and laboratory feeding trials (Hanley 2004; Hanley and Sykes 2011). In June 2009, several hundred seeds were set to germinate separately in large plastic trays (350 mm × 215 mm × 70 mm deep) filled with John Innes No 2 potting compost and maintained in an naturally-lit greenhouse. All species germinated within 3–5 days. When the seedlings were approximately 14-day-old, all above-ground material was harvested, cleaned, and immediately macerated using a mortar and pestle. Macerated material was then divided into 2 g pellets, placed individually in Eppendorf tubes, and frozen until further use.

Eighty snails (*Helix aspersa*) were collected in May 2009 from around Plymouth, UK, and subsequently retained in large plastic containers in an incubator (15°C day/8°C night temperature and 12 h day/night illumination regime) and fed on a mixed diet of lettuce and carrot. Two days prior to the start of olfaction trials (July 2009), snails were moved to a separate container where they were starved. Consequently, all snails experienced the same environmental and dietary conditions for several weeks prior to the start of the experiment, reducing the potential for individual preference and hunger to confound the olfaction trials (Clark et al. 1997; Hanley et al. 2003).

Snail olfaction and seedling selection

A glass Y-tube olfactometer of internal diameter 55 mm with arms 80 mm long (Soham scientific, Ely Cambridgeshire, UK) was used to observe snail response to seedling volatiles. The Y-tube arm ends were connected to 10-l plastic containers containing the test treatments. Air pressure regulators (Caché Instrumentation, Wakefield, UK) were fitted into holes drilled into each container and connected to an air pump. Airflow was set at 500 cm³ min⁻¹. The air entering the set-up came from the room and was not filtered, although experiments were conducted in a sealed laboratory to reduce confounding olfactory stimuli. Illumination was a fluorescent strip light positioned directly above the experimental apparatus.

Following defrosting, a pellet randomly selected from one of the available ‘Test’ species was placed in one of the plastic containers upwind of one of the Y-tube arms. A second pellet of macerated lettuce (‘control’) was placed in the second container to act as a standard against which selection of the ‘test’ species was compared. The position of ‘test’ and ‘control’ pellets in the arms of the Y-tube was randomly assigned on each test. For each test one snail was randomly selected from a pool of 20 snails starved for 2 days prior to the experiment and introduced into the base of the Y-tube. Each snail was then observed for a maximum of 10 min or until it contacted the end of a Y-tube arm. During this time we noted the time taken for each snail to move into one arm of the Y-tube (‘Time to Choose’), the direction chosen on reaching the split (‘Proportion of Choice’), and the time taken to reach the end of the Y-tube arms (‘Time to Sample’). ‘Time to Choose’

and ‘Time to Sample’ were noted as they provided additional information about snail foraging behavior beyond simply noting which pellet was selected. ‘Time to sample’ indicates snail ‘motivation’ to respond towards a favored sample; ‘Time to choose’ indicates the speed to make the decision.

In order to determine whether freezing had any effect on patterns of snail behaviour, we also compared the frequency of selection of frozen and freshly macerated samples of *Trifolium repens*. Trials where snails made a choice within the specified timeframe were conducted 20 times for each test species; trials where the subject snail failed to make a choice within 10 min were discarded. All equipment was thoroughly washed between trials to remove mucus and snails were returned to laboratory culture for a minimum of 4 days prior to starting the next experimental cycle.

Seedling selection and comparison with seedling acceptability

Hanley and Sykes (2011) provide estimates of seedling acceptability to *Helix aspersa* for six of our ‘Test’ species, but acceptability of the remaining three species (*A. vulneraria*, *M. lupulina* and *S. jacobaeae*) was determined in the same experiment (Supplementary material). Seedling acceptability was calculated by comparing consumption of 14-day-old ‘Test’ seedlings relative to consumption of a standard lettuce ‘Control’ in the same test arena. These values were used to calculate an acceptability index (AI) based on the formula given by Fenner et al. (1999). AI ranges between 0 (highly unacceptable) and 1 (highly acceptable) where a value of 0.5 represents equal acceptability to the lettuce control. See Hanley and Sykes (2009) for a full description of the method. Following normalization via arc-sine transformation, we used Pearson’s correlation to compare mean percentage frequency of snail selection for the ‘Test’ species ‘Proportion of Choice’ with AI. We also plotted AI against mean ‘Time to Choose’, and ‘Time to Sample’ for those trials where a snail selected the Y-tube arm leading to the ‘Test’ species.

Results

The frequency of selection of *Trifolium repens* pellets using fresh (‘Test’ selected in 9 of 20 trials) and frozen

(‘Test’ selected in 8 of 20 trials) material was remarkably similar ($\chi^2_1 = 0.102$, $P = 0.794$), and thus we conclude that freezing had no significant effect on snail selection of macerated plant material. When we compared ‘Proportion of Choice’ for the test species with seedling acceptability, we found a highly significant ($r^2 = 0.862$, $F_{2,6} = 18.78$, $P = 0.003$) positive relationship (Fig. 1). Similarly, when we compared mean ‘Time to Choose’, and ‘Time to Sample’ with seedling acceptability, we found strong negative relationships (‘Time to Choose’ $r^2 = 0.90$, $F_{2,6} = 28.12$, $P < 0.001$; ‘Time to Sample’ $r^2 = 0.861$, $F_{2,6} = 18.62$, $P = 0.003$), suggesting that olfactory selection of preferred (i.e. high AI) seedlings occurs more rapidly than for less preferred species (Fig. 2).

Discussion

Our results provide a number of insights into herbivore olfaction. First, as far as we are aware, no previous study has examined herbivore olfactory response to cues released by seedlings from such a range of wild plant species [Carroll et al.’s (2006, 2008) work examined armyworm response to two crop species]. Second, we show that olfactory response is strongly linked to seedling acceptability for nine, sympatric plant species. Third, we found a

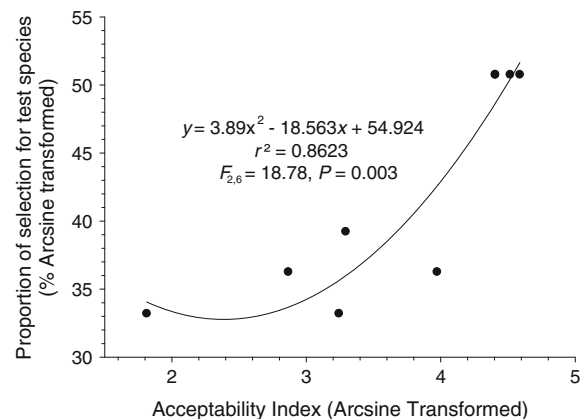


Fig. 1 The relationship between seedling acceptability of nine common temperate grassland plant species and the proportion of times snails (*Helix aspersa*) moved towards a pellet of macerated seedling material in a Y-tube olfactometer in preference to a lettuce control. Acceptability to snails (*Helix aspersa*) was calculated as seedling consumption relative to a standard index species lettuce (*Lactuca sativa*) aged 14 days (see Hanley and Sykes 2009)

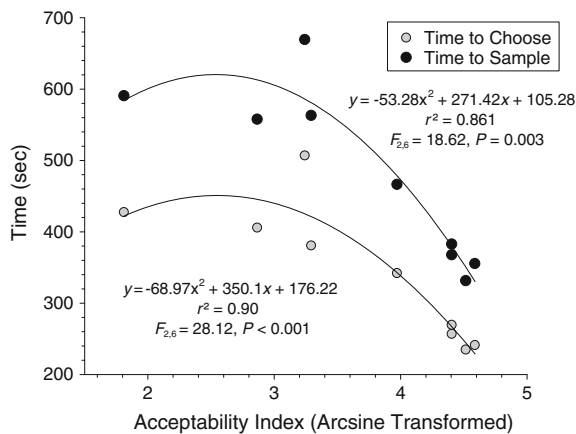


Fig. 2 The relationship between seedling acceptability of nine common temperate grassland plant species and the mean time taken for an individual snail (*Helix aspersa*) to gray circles) move towards a pellet of macerated seedling material ('Time to Choose') in a Y-tube olfactometer and filled circles) the mean time to reach the end of the olfactometer arm ('Time to Sample') in trials where the snail selected the pellet of the grassland test species in preference to a lettuce control. Acceptability to snails (*Helix aspersa*) was calculated as seedling consumption relative to a standard index species lettuce (*Lactuca sativa*) aged 14 days (see Hanley and Sykes 2009)

close relationship between seedling acceptability and the time taken for snails to respond to volatiles released by different seedlings.

We recognize that we did not examine whether olfactory selection was based on the release of attractant or deterrent volatiles, attempt to identify specific volatiles, or relate volatile presence to constitutive plant defenses. Consequently, while high acceptability seedlings were more likely to be selected and selected more quickly, we cannot infer whether selection was based on positive olfactory selection of acceptable species or discrimination against low acceptability species. Additionally, the olfactory responses described here were based on damaged seedlings but the full role played by volatiles cannot be understood without performing trials with intact seedlings. Nonetheless, post-damage volatile emission is important in alerting herbivores to established plants (Garcia-Robledo and Horvitz 2009; Unsicker et al. 2009), and given that seedlings seldom germinate in isolation, herbivore damage to an individual seedling may be important in signaling the relative acceptability of neighboring congeners to other herbivores.

Despite the limitations of this study it nonetheless provides many interesting avenues for further research.

Future studies might investigate the wider role seedling volatiles play in dictating food plant selection by mollusks, both from the point of view of how mollusks influence natural plant communities and also their role as one of the most important economic pests in arable cropping systems (Simms et al. 2006; Nash et al. 2007). Indeed, any insight into the physiology of food plant detection and selection may inform the development of crop cultivars that produce and release deterrent volatiles, or anti-feedants that repel mollusks.

In order to achieve these aims, behavioral studies such as the one conducted here must be conducted alongside systematic identification of the key VOCs involved in olfactory selection. VOCs must then be compared with those constitutive or induced defenses present within plant tissues that deter herbivore feeding. It is also important to establish whether snails detect and respond to particular VOCs or suites of VOCs, and this is best achieved via neurophysiological experiments (Voss 2000; Birkett et al. 2004). Nonetheless, our study provides a basis to expect that seedling selection is at least partly influenced by olfactory cues. Indeed, being able to alert herbivores to defensive capability via VOCs may be particularly important for seedlings because unlike established plants, even a relatively small amount of tissue loss reduces survival, growth, and reproductive potential (Hanley and Fegan 2007). Consequently, a deeper understanding of the physiological and behavioral response of mollusks to seedling volatiles and how they correlate with anti-herbivore defenses can reveal much about broader concepts of herbivore feeding behavior and the evolution of plant defense at this most vulnerable stage of a plant's life history.

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