Arthropod-Plant Interactions
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Immunological dependence of plant-dwelling animals on the medicinal properties of their plant substrates: a preliminary test of a novel evolutionary hypothesis

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Abstract We present preliminary evidence for a novel evolutionary hypothesis, i.e., that animals living on plants high in antimicrobial secondary metabolites could, via drift or selection, evolve weakened immune defenses and an immunological dependence on the antimicrobial properties of their plant substrate and/or the volatile mix in the air surrounding their plant. Animals experience immunological stress during developmental transitions, so we evaluated our hypothesis by testing for an effect of plant substrate on hatching success in the jumping spider *Lyssomanes viridis*, a species that constructs its egg sacs on *Liquidambar styraciflua* leaves. Compared with other sympatric species, *L. styraciflua* is known to be high in potent volatile broad-spectrum antimicrobial compounds, most notably, the monoterpene terpinen-4-ol, a well-studied antimicrobial agent known from tea tree oil. We found that *L. viridis* experience higher hatching success on *L. styraciflua* than on other sympatric species or plastic substrate controls and that *L. viridis* has a chemically mediated preference for this plant. In contrast to other spiders' compact, densely woven egg sacs, *L. viridis*' egg sacs are sparsely woven and the eggs widely spaced, maximizing the eggs' surface area exposed to volatiles coming off the leaf. Similar architectures exist in distantly related taxa, suggesting convergent evolution. Our theoretical framework and preliminary results open up an unexplored frontier—the possibility that any number of plant-dwelling species may depend upon on the antimicrobial properties of the plant tissues they inhabit.

Keywords Chemical ecology · Arthropod–plant interactions · Insect–plant interactions · Plant–insect–microbe interactions · Medicinal plants · Development · Fungus · Bacteria · Virus · Infection

Introduction

Plant secondary metabolites are compounds manufactured by plants that are embedded in their tissues, some of which are slowly released into the atmosphere, and whose functions often include protection from herbivory and invasion by bacteria, fungi, and viruses (Wink 2010). Humans have been co-opting the prophylactic and medicinal properties of plant materials to promote their own survival and wellness for thousands of years (Petrovska 2012), and animal species from disparate taxonomic groups have evolved to do the same. Certain mammals, birds, and insects have been shown to supplement their nests or rub their bodies with plant materials high in secondary compounds, apparently to stave off various types of infections and parasites (Gompper and Hoylman 1993; Baker 1996; DeJoseph et al. 2002; Zito et al. 2003; Chapuisat et al. 2007; Simone et al. 2009; Simone-Finstrom and Spivak 2012; Dubiec et al. 2013).

Although our knowledge of animal uses of medicinal plants comes thus far from observations of animals that actively gather plant material, a more rudimentary, and

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thus possibly more common, strategy could be for a population or species to evolve a more passive dependence on the antimicrobial properties of a plant(s) it already commonly inhabits and reproduces on. Such a population or species could already commonly inhabit a particular plant due to a preference its architecture, for example, or for historical/ecological reasons such as low plant species diversity. In any case, natural selection could favor a weakening of an animal's immune defenses if its plant substrate, and/or the volatile mix in the air surrounding its plant, effectively fought the animal's immune battles for it, since this would presumably free up resources that could be more beneficially allocated elsewhere. A weakening of an animal's immune defenses could also arise via genetic drift due to relaxed selection on an animal's immune defenses, without concomitant selection for a reallocation of resources. In either case, such an evolved immunological dependence would likely often result in, or be accompanied by, selection for a behaviorally mediated preference, or a strengthening of a preexisting preference, for said plant(s) if a strong preference did not already exist. We would expect plant chemical defenses to be particularly beneficial during sensitive developmental transitions such as egg hatching and molting, periods of immunological stress (An et al. 2012) during which animals experience high mortality (Gardner 1965; Horner and Starks 1972; Ruppert et al. 2004).

The idea that plant secondary compounds could potentially help plant-dwelling animals fight infection was hinted at nearly 30 years ago by Faeth (1986) and Faeth and Bultman (1986), who found that leaf miners suffered lower mortality from microbial infections on previously damaged leaves (which were higher in condensed tannins), and on leaves with tannic acid artificially applied to them, than they did on undamaged or control leaves. However, despite the apparent medicinal benefits of damaged leaves, adult females were found to avoid ovipositing on such leaves, and eggs that were laid on damaged leaves attained lower pupal weights and were more likely to be parasitized. Under natural conditions, the net effect of all these factors was higher survivorship on undamaged leaves (Faeth 1986; Faeth and Bultman 1986). The possibility of an evolved immunological dependence on the chemical profile of undamaged leaves, which may have contained adequate chemical defenses to exert some (albeit lesser) medicinal effect, remains unexplored to this day.

We chose the jumping spider *Lyssomanes viridis* to test for the possibility of an evolved dependence on the antimicrobial defenses of a plant because we knew that it already met several assumptions of our theoretical framework. Through our own extensive sampling, using a beating sheet, of all common species of trees within *L. viridis*' habitat in the North Carolina Piedmont, we have found that *L. viridis* is far more abundant on a tree species high in volatile secondary metabolites (*Liquidambar styraciflua*, or "sweet gum," Wyllie and Brophy 1989) during its breeding season than on any other sympatric tree species common to the North Carolina Piedmont. We have also found that *L. viridis* eggs fail to survive the hatching process when the egg sac is constructed on a plastic substrate in the laboratory, whereas other salticid species' (*Thiodina sylvana*, *Phidippus otiosus*) eggs hatch normally in the laboratory; this suggests there is nothing peculiar to our laboratory environment that prohibits egg or postembryo survival. *L. viridis*' egg sac architecture is somewhat unusual in that the eggs are spaced apart from one another in a diffuse silken net suspended a few millimeters below a leaf's surface (Fig. 1) (Richman and Whitcomb 1981; Hallas and Jackson 1986). This architecture likely increases the thickness of the boundary layer (i.e., stagnant air around the leaf) to envelop and permeate the egg sac in a way similar to how leaf pubescence increases boundary layer thickness (Schuepp 1993). Other jumping spider species' eggs tend to be clumped together in densely woven silken nests (Richman and Jackson 1992), which are far less permeable to air and often impermeable even to liquid water (Rovner 1987; Gunnarsson 2000). It is from these latter, more typical, nests that we have successfully hatched eggs in the laboratory. It thus seems possible that *L. viridis*' egg sac architecture, as an extended phenotype, has evolved to maximize the exposure of its eggs to volatile compounds that are concentrated in the boundary layer of its host plant.

Sweet gum leaf oil is packed with volatile monoterpenes, over ten of which have proven antimicrobial activity, and is particularly high in the monoterpene terpinen-4-ol, the broad-spectrum antimicrobial agent known from tea tree oil (*Melaleuca alternifolia*) (Wyllie and Brophy 1989; Carson and Riley 1995; Dorman and Deans 2000; Hinou et al. 1989; Klein et al. 2013; Marei

Fig. 1 Female *L. viridis* guarding her hatching egg sac

et al. 2012; Naigre et al. 1996; Pattnaik et al. 1997; Mondello et al. 2006; Pinto et al. 2006; Reichling et al. 2006; Tirillini et al. 1996). Interestingly, southern magnolia (*Magnolia grandiflora*), a tree on which *L. viridis* has been observed to reproduce in northern Florida (Richman and Whitcomb 1981), but is uncommon in North Carolina (Khela 2014), also has leaf oil with demonstrated antimicrobial activity (Guerra-Boone et al. 2013). It, too, is high in volatile monoterpenes, several of which are also present in sweet gum, as well as sesquiterpenes; it does not, however, contain the broad-spectrum terpinen-4-ol (Fan et al. 2009; Guerra-Boone et al. 2013; Jimenez-Medina et al. 2007). Terpenes in general tend to disrupt membranes, causing cell leakage and/or modulation of membrane proteins and receptors, leading to cell death (Sikkema et al. 1994).

We first tested whether *L. viridis* prefer the chemical cues of sweet gum by challenging them with a series of binary choice tests in which they were allowed to choose between the chemical cues of sweet gum versus four other sympatric tree species: American holly (*Ilex opaca*), southern magnolia (*Magnolia grandiflora*), white oak (*Quercus alba*), and American beech (*Fagus grandifolia*). The headspace of white oak has been characterized and contains some terpenes, but none whose antimicrobial activities are known to be as broad spectrum or as powerful as terpinen-4-ol in sweet gum (Pearse et al. 2013). Neither the leaf oils nor the headspace of American holly or American beech have been characterized. Of the trees we tested, sweet gum and magnolia are the only ones that emit a pungent odor (as perceived by humans) (Wyllie and Brophy 1989). To determine whether *L. viridis* eggs experience greater hatching success on sweet gum than they do on other sympatric tree species, we had females lay their eggs on sweet gum saplings and on saplings of three of the above four sympatric tree species, and on polyvinyl chloride (PVC) plastic as a control, in an environmental chamber programmed to replicate daily fluctuations in temperature and humidity in the spiders' natural habitat. On pliable leaves like those of sweet gum and white oak (but not on southern magnolia or American holly), the tautness of the silk threads making up the flat egg sac slightly enhances leaf concavity, so we also included two PVC treatments—concave versus flat pieces of PVC—to test whether the concavity of leaves, and/or the female's ability to manipulate leaf concavity during egg sac construction, could be having an effect on egg viability. Saplings were transplanted from *L. viridis* habitat after they had leafed out in an effort to maintain semi-realistic bacterial populations and abundances on the plants used in experiments.

Methods

Subjects and housing

For the leaf preference experiments, immature *Lyssomanes viridis* (Walckenaer 1837) were collected by beating sweet gum trees (*Liquidambar styraciflua*) along the Black Creek Greenway (35°49.3′N, 78°47.1′W) in Wake County, North Carolina, USA, and in Jordan Lake State Recreation Area (35°50.0′N, 78°58.0′W) in Chatham County, North Carolina, USA, in August 2009, 2011, and 2012. For the egg viability experiments, immature *L. viridis* were collected by beating American holly trees (*Ilex opaca*), an evergreen species that we have observed *L. viridis* to commonly overwinter on, in William B. Umstead State Park (35°50.5′N, 78°45.4′W) and in Eno River State Park (36°3.4′N, 78°58.8′W), in an area spanning Durham and Orange Counties, North Carolina, USA, in March and April. Housing conditions were as described previously (Tedore and Johnsen 2013). Photoperiod was regulated by a timer that followed the natural outdoor photoperiod in the North Carolina Piedmont.

Leaf preference experiments

The trees selected to be tested against sweet gum in binary choice tests were southern magnolia [*Magnolia grandiflora* (Magnoliaceae)], American holly [*Ilex opaca* (Aquifoliaceae)], white oak [*Quercus alba* (Fagaceae)], and American beech [*Fagus grandifolia* (Fagaceae)]. Previous work has demonstrated that *L. viridis* in Florida can and does reproduce on southern magnolia (Richman and Whitcomb 1981). Although this species can be found in North Carolina, it is rare and generally planted, and we have not sampled it regularly. However, when we have sampled it, we have found *L. viridis* on its leaves. American holly was chosen because we find *L. viridis* at high densities on its evergreen leaves during the wintertime when deciduous sweet gum leaves are unavailable. White oak was chosen because we occasionally find juvenile *L. viridis* on this species, which suggests that it is not particularly repellent to them. American beech was chosen because it is common in *L. viridis*' habitat, but we do not find *L. viridis* on it.

Experimental trials were conducted using juvenile spiders between September and November, and leaves for experiments were harvested fresh each morning from the Black Creek Greenway (35°49.3′N, 78°47.1′W) in Wake County, North Carolina, USA, and from the Duke University campus (36°0.1′N, 78°56.6′W) in Durham County, North Carolina, USA. Spiders were given four *Drosophila melanogaster* and a light misting of filtered water daily after experimental trials were complete for the day, and at least 1 h before their lights turned off.

Contact + airborne chemical cues

Here we presented spiders with standard-sized leaf rectangles to test for a chemically mediated (either contact or airborne) preference for the leaves of sweet gum over other sympatric tree species. We cut out 5.0 × 2.5 cm leaf rectangles of each species for each test. The rectangles were cut such that the midrib was oriented down the center of the long axis of the rectangle in all species. Leaf samples never included visibly damaged, diseased, or senescent tissue. *L. viridis* prefers to cling to the undersides of leaves, so the two samples were affixed with transparent Scotch tape onto the inside of the lid of a clean 10 × 10 × 10 cm clear plastic box as shown in Fig. 2. Tape was oriented to flatten out leaves with any natural concavity, and to prevent the drooping of leaves as they began to wilt. The orientation and placement of the tape was always the same for each of the two choices presented in a given test.

Each spider was placed on the floor of a 10 × 10 × 10 cm experimental chamber and allowed 3 h to walk up the walls and make a choice between the two leaf samples. Each spider received its own fresh pair of leaf rectangles to choose from. Experimental chambers were surrounded on three sides by white paperboard, and were illuminated from above by two full-spectrum (including ultraviolet) fluorescent mercury vapor tubes (T8, 32 Watt, 48 inch, Duro-Test Lighting's Vita-Brite, Philadelphia PA, USA). Each chamber was oriented such that the long axes of the leaf rectangles were perpendicular to the long axes of the overhead fluorescent lights. To control for potential side biases, we randomly assigned half of the subjects to be presented with *L. styraciflua* on one side of their experimental chamber, and the other half to be presented with *L. styraciflua* on the other side of their experimental chamber.

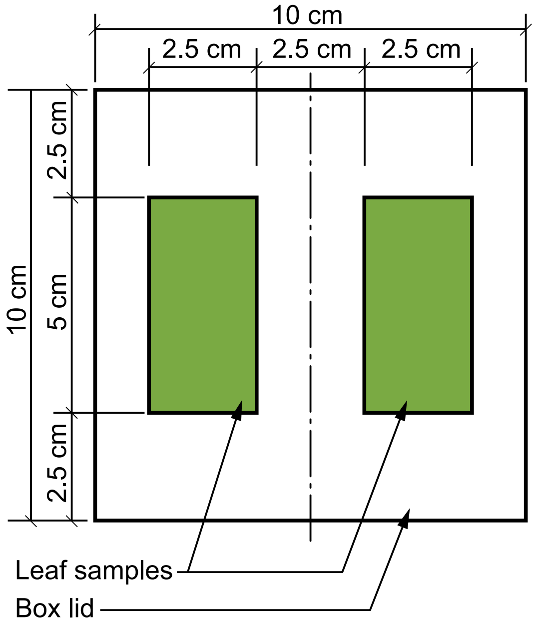

Fig. 2 Placement of leaf samples on experimental arena lid

Each spider was allowed to wander about its experimental chamber for 3 h before we checked which leaf it was clinging to. Spiders that were not on either leaf were retested the following day. Spiders that did not make a choice on the second day were retested a third (and final) day. On each of these successive trials, sweet gum was presented on the opposite side of the experimental chamber as it had been presented on the previous day.

To ensure that spiders were not using subtle visual cues, such as color, brightness, or venation, to discriminate between leaf species, we ran a control for three of the four tree species that sweet gum had been tested against above. Spiders were given a choice between leaf rectangles as before, but this time, the samples were taped to the outside of the box instead of the inside. The box lids were transparent, allowing the spiders to see the leaves, but not to taste, touch, or smell them. Using this methodology, sweet gum was again tested against American beech, white oak, and southern magnolia. As a final control, we then gave spiders a choice between a sweet gum leaf rectangle taped to the inside versus outside of the box. This control was designed to ensure that the preferences we observed in the above experiments were a result of attraction to a preferred leaf, rather than repulsion from a disfavored one.

Airborne chemical cues only

To determine what the role of airborne chemical cues alone might be in mediating leaf preference, we taped leaf rectangles to the insides of experimental chamber lids, as above, but this time prevented spiders from physically touching the leaves. We accomplished this by stretching a fine fabric mesh over a 152-mm embroidery hoop and placing it between each experimental chamber box and its lid. This left a space between the mesh and the leaf of about 5 mm. As before, each spider was allowed to explore the chamber for 3 h before we scored which of the two leaves it was perched under. The spider had to be perched on the mesh directly under a leaf for a choice to be recorded. Spiders that did not make a choice repeated the experiment a maximum of three times, as in the above experiments.

Egg viability experiments

Here we tested whether the species of leaf a female laid her eggs on had an impact on the viability of her eggs. Spiders to be mated were collected immature, as described above. Prior to their terminal molt, spiders received eight *D. melanogaster* and a light misting of filtered water twice per week. Because adult females face high energy demands for producing eggs, as evidenced by their rapid consumption of any flies we gave them, we increased feeding frequency upon maturation for females, giving them eight *D. melanogaster* and a light misting of water daily. During the week leading up to a male being paired with a female, he was given four *D. melanogaster* and a light misting of water daily. This provided males with consistent access to food without overrunning their enclosures with uneaten flies.

Virgin male and female spiders were randomly paired and allowed to copulate in May and June. Females that failed to mate with the male they were paired with were presented with a different male on the following day. All females were mated between 1 and 20 days past their terminal molt. Males were mated between 6 and 40 days past their terminal molt. After mating, females were visually inspected daily for the development of eggs in the abdomen. When a female *L. viridis* is a few days away from oviposition, the outlines of individual eggs become visible through her translucent abdominal cuticle, and she stops eating (CT, personal observation). When this happened, females were randomly assigned and moved to one of six experimental treatments in one of two replicate environmental chambers. Treatments included sweet gum, southern magnolia, American holly, and white oak saplings, and curved and flat pieces of PVC. The curved and flat PVC treatments were designed not only to function as negative controls, but also to test the effect of leaf concavity on egg viability.

Saplings of each tree species were transplanted, after they had leafed out, from the Duke University campus in Durham County, North Carolina, USA (36°0.2′N, 78°56.7′W), and from private property adjacent to the Eno River State Park in Orange County, North Carolina, USA (36°5.1′N, 78°59.9′W), in April and May 2012. Saplings were fertilized with half-strength Hoagland solution (see Suppl. 1 for composition) once per week.

Curved pieces of PVC were constructed from Harvel Clear polyvinyl chloride (PVC) pipe (Georg Fischer Harvel LLC, Easton PA, USA) five cm in diameter (inside diameter). Pipe was cut crosswise into 15 cm lengths and then cut in half lengthwise. Flat sheets of the same material were cut to dimensions that gave them the same surface area as the curved pieces, i.e., if it were possible to flatten out the curved pieces, then these flattened pieces would have had the same dimensions as the pieces that were flat to begin with (15 cm × 8 cm). Both the pipe and flat sheets of PVC were transparent and purchased from ALSCO Clear PVC Pipe and Fittings in Lithia Springs GA, USA. The thickness of the plastic itself in the curved sheets was 3.9 mm and that of the flat sheets was 3.2 mm. To shield the eggs from direct overhead light, we covered the upper surface of each PVC piece with two layers of PVC static cling film (Grafix Plastics, Cleveland OH, USA). The lower layer of cling film was dark green to give the spiders a semi-realistic and dark place to hide and lay their eggs. The upper layer was white to reflect overhead light in order to prevent heating by the absorption of radiant energy. PVC pieces were clamped to a ring stand at the same height from the ground as the saplings.

To force a female to stay on the tree or PVC piece she was assigned to, each tree and PVC piece was enclosed with mosquito netting. To prevent the weight of the netting from warping the shape of each sapling and its leaves, a wire cage was placed around each tree to provide structure. The netting was removed after the female had laid her eggs.

During the environmental chamber phase of the study, females were provided with ten *D. melanogaster* daily by tapping flies out of a vial onto the upper surface of the leaf or PVC piece the spider was resting under. This was repeated daily until 20 days past the female's oviposition date, at which time the leaf on which the egg sac had been spun on was removed and the eggs/spiderlings assessed under a microscope. Unhatched and partially hatched eggs that died before completing hatching were visibly rotten (i.e., brown) by this time point. Eggs that hatched into viable, mobile spiderlings never looked brown like this at any stage of hatching. Five egg sacs were laid on sweet gum, seven on southern magnolia, four on American holly, four on white oak, fourteen on curved PVC, and eleven on flat PVC.

To program the chambers, we analyzed weather data collected from a long-term NSF-funded field study conducted at the Duke University Forest FACE facility (Ward et al. 2013). We verified that the field site was *L. viridis* habitat by beating sweet gum trees at the site and finding several individuals. Weather data had been collected three meters above ground in Ring 1 of the FACE site study area (35°58.7′N, 79°5.6′W) for 3 years, from 2004 to 2006. We calculated three-year mean temperatures and relative humidities at half-hour intervals throughout the 24-h cycle, averaged across all the days in June (i.e., the height of the breeding season). The environmental chambers were programmed to match these conditions, modulating temperature and humidity every 30 min throughout the 24-h cycle, with the same cycle being repeated daily. The light cycle in the chamber was programmed to follow the daily light cycle in central North Carolina. Photosynthetically active radiation (PAR) at the average top of canopy height in the chamber was 400 μmol photons m^{-2} s^{-1}. We split the treatments evenly among two identically programmed chambers so that approximately the same number of individuals was run in each treatment in each chamber.

Results

Leaf preference experiments

Contact + airborne chemical cues

In all binary choice tests in which *L. viridis* could receive both contact and airborne chemical cues from sweet gum and the alternate species, *L. viridis* chose the sweet gum leaf rectangle over the alternate species (two-tailed binomial exact test for sweet gum versus (1) American beech: $N = 12$, $P = 0.00049$; (2) white oak: $N = 24$, $P = 0.023$; (3) southern magnolia: $N = 35$, $P = 0.0060$; (4) American holly: $N = 18$, $P = 0.031$) (Fig. 3). When given a choice between leaf rectangles they could see, but could not receive any chemosensory cues from, *L. viridis*' preference for sweet gum disappeared (two-tailed binomial exact test for sweet gum versus (1) American beech: $N = 23$, $P = 0.41$; (2) white oak: $N = 22$, $P = 1.0$; (3) southern magnolia: $N = 21$, $P = 0.38$) (Fig. 3). When *L. viridis* were given a choice between sweet gum leaf rectangles they could and could not receive chemosensory cues from (i.e., inside vs. outside the box lid), both of which they could see, they chose the leaf rectangle that they could receive chemical cues from (two-tailed binomial exact test, $N = 24$, $P = 0.023$) (Fig. 3).

Airborne chemical cues only

When *L. viridis* could receive airborne, but not contact, chemical cues in binary choice tests, they preferred sweet gum over white oak (two-tailed binomial exact test, $N = 13$, $P = 0.0034$), but preferred southern magnolia over sweet gum ($N = 17$, $P = 0.013$). Spiders showed no preference in binary choice tests between sweet gum and American beech ($N = 14$, $P = 0.79$) or between sweet gum and American holly ($N = 18$, $P = 1$) (Fig. 4).

Egg viability experiments

Egg hatching and development into mobile spiderlings tended to halt at different stages on different treatments. We classified four different stages of egg hatching. In the first, termed "featureless," eggs never began to hatch or to show any visible signs of development under a dissecting microscope. In the second, termed "early hatching," eye pigmentation became visible through the chorion and, in some cases, the chorion cracked open and it was possible to see a spiderling with its legs stuck together and folded against its body. When the legs became fully separated from the body and from each other, we considered this a third stage of development, termed "late hatching." Many spiderlings never became mobile after this stage and

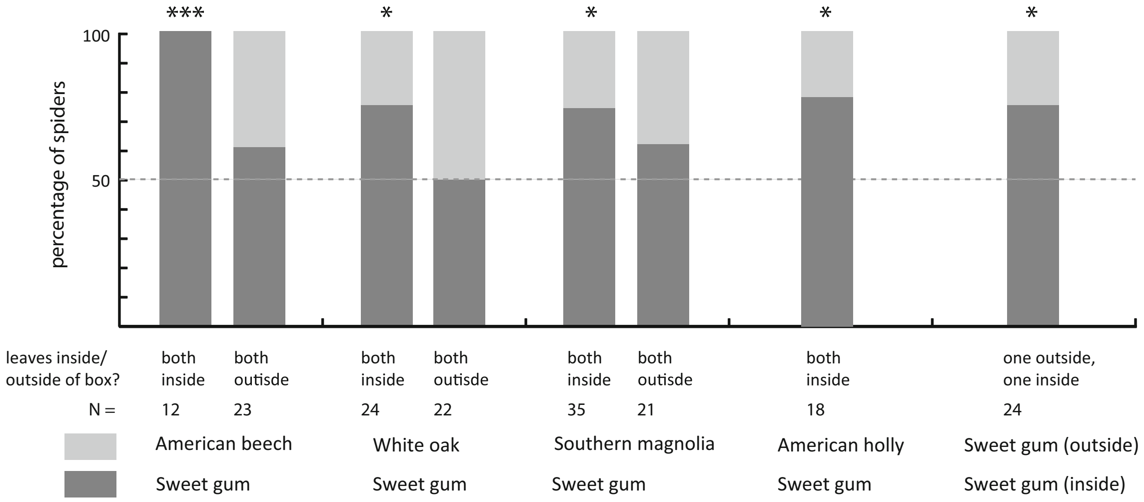

Fig. 3 Binary choice tests between cutout rectangles of sweet gum leaves (*dark gray* portion of each bar) versus cutout rectangles of four other tree species (*light gray* portion of each bar). The two leaf samples in each test were taped to either the inside or the outside of the clear plastic lid of the experimental arena, the latter treatment serving to test for the use of visual cues. In another test, spiders were given a choice between a sweet gum leaf rectangle taped inside versus outside the lid in order to determine whether spiders were actually attracted to sweet gum, or simply repelled by the other species. The *dotted line* shows chance expectations. An *asterisk* above a bar indicates that spiders chose the sweet gum leaf square inside the lid significantly more often than the alternate option (*<0.05, ***<0.0005). Each bar corresponds to a separate experiment; to facilitate comparisons, the "both inside" versus "both outside" bars for each species pair have been grouped together in the figure

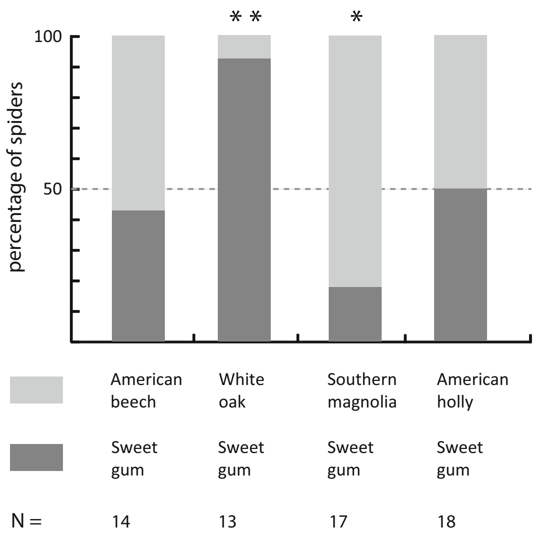

Fig. 4 Binary choice tests between cutout squares of sweet gum versus three other tree species. Leaves were taped to the inside of the lid, but spiders were prevented from touching the leaves by a thin layer of mesh suspended 5 mm below the leaf squares. The *dotted line* shows chance expectations. An *asterisk* indicates that spiders chose one leaf species significantly more often than the alternate option (*<0.05, **<0.005)

simply died without ever walking around. Thus, we named a fourth stage, termed "walking," to designate spiders that actually became mobile and started walking around (Fig. 5). Each egg sac was scored for the spiderling that showed the most advanced stage of development.

To increase our statistical power, we combined the "featureless" and "early hatching" stages into one group we termed "unviable," and the "late hatching" and "walking" stages into another group we termed "semi-viable" for the purposes of statistical testing. Using a two-tailed 2 × 2 Fisher's exact test, we found that eggs were significantly more likely to be semi-viable on sweet gum than on white oak ($P = 0.0079$), curved PVC ($P = 0.0048$), or flat PVC ($P = 0.00023$). Eggs were also more likely to be semi-viable (albeit not significantly so) on sweet gum than on southern magnolia ($P = 0.081$) or American holly ($P = 0.17$). Eggs were not significantly more likely to be semi-viable on curved than on flat PVC plastic (0.23) (Fig. 6).

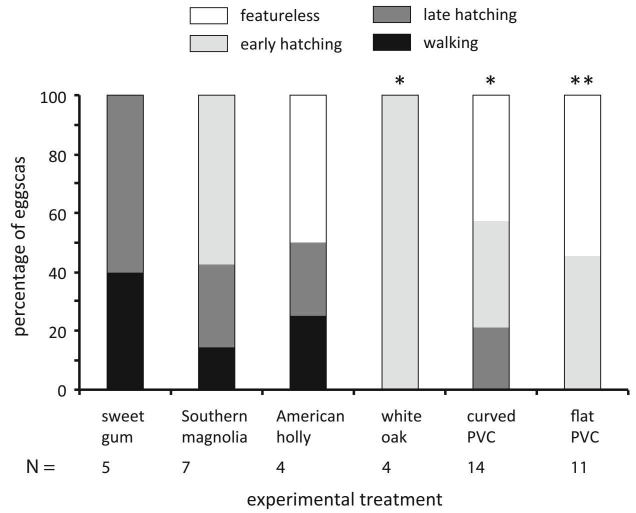

Fig. 6 Hatching success on four different tree species and on curved versus flat sheets of PVC. Each egg sac was scored for the spiderling attaining the furthest phase of development in that egg sac. An *asterisk* over a bar indicates that a greater number of egg sacs made it to the late hatching or walking phase on sweet gum than on the alternate treatment listed below that bar (*<0.01, **<0.001)

Discussion

Our results provide preliminary support for our hypothesis that *L. viridis* has evolved a dependence on, and a corresponding preference for, chemical properties of sweet gum for successful egg hatching. When both contact and airborne chemical cues were available, *L. viridis* demonstrated a clear preference for sweet gum leaves over all four of the other species tested (Fig. 2). The fact that *L. viridis* also preferred sweet gum affixed to the inside of the arena lid over sweet gum affixed to the outside of the arena lid indicates that *L. viridis*' preference for sweet gum in previous trials was not simply a result of it being repelled by the alternate species while at the same time being attracted to visual cover. However, when exposed to airborne cues only, *L. viridis* distinguished sweet gum from only two of the four species tested, and for one of those species, actually preferred the alternate species (southern magnolia) over sweet gum (Fig. 3). The fact that *L. viridis* preferred the odor of sweet gum over that of white oak, but had no

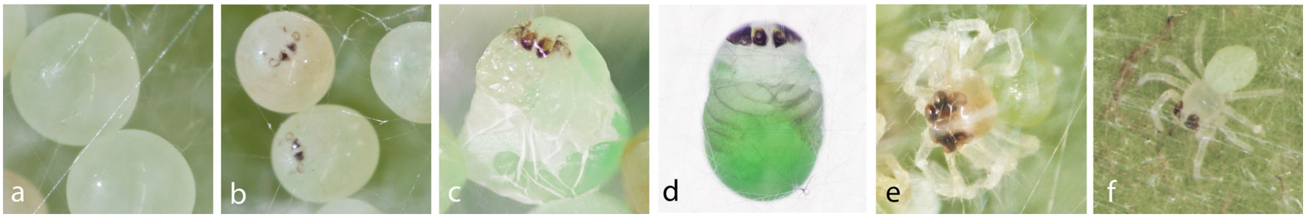

Fig. 5 Different stages of hatching: **a** featureless, **b–d** early hatching, **e** late hatching, **f** walking

preference for the odor of sweet gum odor over the odors of American holly or American beech, suggests that *L. viridis* is repelled by volatiles of white oak, rather than attracted to the volatiles of sweet gum. That said, it is possible that our cutting of the leaves into standardized shapes could have altered the volatile chemical profiles of these species. However, if we take our results at face value, *L. viridis*' olfactory preference for southern magnolia, combined with its apparent lack of olfactory preference for sweet gum, suggests that plant odor may be a more important cue to *L. viridis* in regions where southern magnolia is common, such as in Florida (Maisenhelder 1970), than it is in the northern parts of *L. viridis*' range, where sweet gum is its more common host species, as in North Carolina. It would appear that in the northern parts of its range, *L. viridis* must find its more common and preferred host, sweet gum, by walking across different trees until, by chance, it steps onto one with the correct contact chemical cues. This is an interesting problem, since, as described in the Methods, *L. viridis* juveniles overwinter on the evergreen American holly and must relocate sweet gum in the spring when its leaves emerge. For an animal that cannot fly, and cannot control the direction the wind takes it when it balloons, finding an appropriate host species by touch would seem to be a rather inefficient search strategy.

L. viridis eggs advanced to significantly further stages of hatching on sweet gum trees than on white oak or PVC. They also seemed to fare better on sweet gum than on southern magnolia or American holly, although these differences were not significant. This indicates that *L. viridis* has evolved a dependence on certain properties of sweet gum leaves that are absent in white oak and PVC plastic. Differences in volatile secondary metabolite profiles between the species may explain the differential hatching success that we observed. As outlined in the Introduction, the most concentrated constituent in sweet gum leaf oil is terpinen-4-ol, a volatile monoterpene well known from tea tree oil (*Melaleuca alternifolia*) to have powerful broad-spectrum antimicrobial activity. Tea tree oil, and terpinen-4-ol in particular, has generated much interest in the dermatological research community as a topical antimicrobial agent for skin infections (reviewed by Pazyar et al. 2013). Pinenes, abundant in both sweet gum and southern magnolia, have also been shown to possess antimicrobial activity, but a given volume of a pinene is not as potent or as broad spectrum as the same volume of terpinen-4-ol (Dorman and Deans 2000). This may explain the intermediate welfare of eggs laid on southern magnolia, which does not appear to contain terpinen-4-ol (Fan et al. 2009; Guerra-Boone et al. 2013; Jimenez-Medina et al. 2007). Although *L. viridis*' ontogeny has been formally described on southern magnolia in Florida, which is in the southern part of its range, it is unclear whether it also reproduces on sweet gum that far south. In future, we hope to survey and compare *L. viridis*' host plant use in Florida with that in North Carolina. It would also be interesting to assess whether the microbes that infect *L. viridis* eggs in Florida differ from those that infect them further north, and if so, whether *L. viridis*' preferences for plants with optimal secondary metabolite profiles in different regions varies accordingly.

Although *L. viridis* progressed to later stages of hatching on sweet gum than they did on other tree species and on PVC, many still did not make it to the completely viable walking phase. Although eggs were more likely to progress to the late stages of hatching on sweet gum, many spiderlings died after their legs separated from their body and from each other, and they never began to walk about. Given how closely our environmental chambers mimicked natural conditions, it is difficult to come up with a plausible explanation as to why this may have happened. One possibility is that there is some essential nutrient, i.e., a precursor to one or more secondary metabolites, in sweet gum's natural habitat that was lacking, or simply not concentrated enough, in the fertilizer provided in captivity. Careful measurements of soil nutrients and volatiles emitted by intact leaves still attached to a plant in its natural habitat, compared with a potted plant in an environmental chamber, may provide more clues as to why *L. viridis* eggs fared poorly in captivity. Chemical differences between the leaves of juvenile versus mature trees may also have played a role.

In conclusion, it seems possible that volatile broad-spectrum antimicrobial secondary metabolites could have been responsible for *L. viridis*' higher hatching success on sweet gum and that *L. viridis*' diffuse egg sac architecture may have evolved to exploit the prophylactic effects of volatile compounds concentrated at the leaf surface of species with strong chemical defenses. Although *L. viridis*' egg sac structure is unusual, similar architectures do exist, not only in at least a few other lyssomanines (Eberhard 1974; Jackson 1990; Bertner 2010), but also in the distantly related plexippoid salticids, as can be seen in *Viciria praemandibularis* (Hill 2010), *Telamonia festiva* (Yeo 2014), and *Epeus* spp. (Bay 2012; Javagal 2012; Chuan-Yuan 2008). These analogous architectures in distantly related species suggest an independent derivation of this trait in different taxa, and suggest that *L. viridis*' potential evolved dependence on plant volatiles may not be a peculiarity of one particular species or taxonomic group. Indeed, it is possible that a dependence on the antimicrobial effects of plant secondary metabolites during challenging developmental transitions may have evolved in any number of plant-dwelling species, and in particular, the myriad taxonomic groups that lay their eggs on or in leaves.

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