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Investigating a Photolytic Metabolite in the Nocturnal Grasshopper *Schistocerca ceratiola* (Orthoptera: Acrididae)

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Abstract

The rosemary grasshopper, *Schistocerca ceratiola* Hubbell and Walker (Orthoptera: Acrididae), is unusual because it is one of only two known species of monophagous grasshoppers in North America and is nocturnal. *S. ceratiola* is a specialist herbivore of Florida rosemary, *Ceratiola ericoides* Michuax. Ceratiolin, the most abundant secondary metabolite in the plant, represents the only known example of a photoactivated allelopathic compound. Ceratiolin decomposes in sunlight to yield hydrocinnamic acid and other undescribed breakdown products. Due to the monophagous behavior, ceratiolin is ingested every time *S. ceratiola* feeds. Coupled with the nocturnal behavior of *S. ceratiola*, a connection to the photolytic properties of ceratiolin warrants investigation. We hypothesize that the breakdown products of ceratiolin represent potentially noxious compounds and *S. ceratiola* may exhibit nocturnal feeding behavior to avoid ingesting ceratiolin in sunlight where it readily decomposes. To our knowledge, this is the first chemical ecology study of a specialist herbivore of *C. ericoides* and a possible connection between the nocturnal behavior of *S. ceratiola* and ceratiolin. Qualitative analysis by liquid chromatography and tandem mass spectrometry was performed on the regurgitant, hemolymph, and frass of *S. ceratiola* to determine whether ceratiolin is confined to the gut or if it transports to the hemocoel. We also analyzed samples for the presence of hydrocinnamic acid to determine whether ceratiolin decomposes after it has been ingested. We detected ceratiolin in the regurgitant and frass. We did not detect hydrocinnamic acid in the regurgitant, hemolymph, or frass. Our results indicate that ceratiolin is confined to the grasshopper gut. We discuss more than one opportunity for future chemical ecology studies in this system.

Keywords: *Schistocerca ceratiola*, *Ceratiola ericoides*, monophagy, Florida rosemary, rosemary grasshopper

The staggering diversity of insects is attributed, in part, to phytophagy and the specificity with which most herbivorous insects feed (Mitter et al. 1988, Jaenike 1990, Janz et al. 2006, Wiens et al. 2015). The majority of phytophagous insects are considered oligophagous, feeding on plants from a few select families, with a notable exception to this trend being the grasshopper family Acrididae (Orthoptera) (Jaenike 1990, Bernays 1998). Unlike most phytophagous insects, grasshoppers are often polyphagous, feeding on many plants in many families. Previous studies have classified approximately 60% of all known grasshopper species as polyphagous, and another 25% as graminivorous, yet few species are classified as monophagous (Bernays 1991, Chambers et al. 1996, Chapman and Sword 1997, Picaud et al. 2003).

The relationship between insect herbivores and plants is formed by perception of the specific phagostimulants, deterrents, and the nutritional quality of a plant by the insect (Otte 1975; Cates 1980; Chapman et al. 1988; Bernays 1991, 1998; Bernays and Chapman 2000). Grasshoppers are capable of associative learning wherein, even if a potential host plant is determined to be palatable by the grasshopper, postingestive effects of noxious secondary compounds in the plant will alter future host plant selection (Bernays and Chapman 2000). Therefore, investigating the chemical ecology of a grasshopper with a restricted diet provides a rare opportunity to examine a principal driver of specialized herbivory in a mostly polyphagous group (Otte and Joern 1976). In this study, we examine

a monophagous grasshopper endemic to central Florida and the physiological fate of a unique secondary metabolite produced by its host plant.

Running lengthwise through peninsular Florida are upland ridges with remnants of the early Pleistocene era ecosystem when the xeric shrubland known as 'Florida scrub' was prevalent in the southeastern United States (Lamb et al. 2006, Trapnell et al. 2007, Wheeler 2012). The ridges remained above water in the late-Pleistocene glacial melt and now encompass the last stands of ancient Florida scrub (Trapnell et al. 2007, Wheeler 2012). These areas are of great ecological importance because they are home to over 50 species of endemic arthropods and at least 40 species of endemic plants (Deyrup 1989, Deyrup 1990, Fischer et al. 1994, Lamb et al. 2006). One of these ecologically significant organisms is an aromatic shrub, *Ceratiola ericoides* Michaux (Ericaceae, formerly Empetraceae), colloquially called Florida rosemary due to its resemblance to the edible herb.

In permitting conditions, hundreds of individual *Ceratiola* bushes will conspicuously dominate an area usually near one of Florida's numerous ponds and lakes (Hubbell and Walker 1928, Menges and Hawkes 1998). The striking feature of these vegetative patches is the lack of competitive growth from neighboring vegetation (Johnson 1982, Williamson et al. 1992, Menges and Hawkes 1998, Smith and Capinera 2005, Wheeler 2012). The amount of exposed sand in the localities dominated by Florida rosemary has led these areas to be commonly referred to as rosemary balds. The lack of competitive plant growth in rosemary balds is due to allelopathic chemicals in the soil that are leached from the leaves of *C. ericoides* by rainwater and inhibit the germination and growth of many other plants (Johnson 1982, Tanrisever et al. 1987, Williamson et al. 1992, Fischer et al. 1994). The inhibition of grass growth is particularly important because Florida rosemary is susceptible to the frequent fires characteristic of Florida scrub and the grasses provide fuel for the fires in the dry season (Johnson 1982, Tanrisever et al. 1987, Williamson et al. 1992, Fischer et al. 1994, Menges and Hawkes 1998).

A secondary metabolite unique to *C. ericoides*, a dihydrochalcone named ceratiolin, is broken down by sunlight to yield hydrocinnamic acid (HCA), the phytotoxic compound responsible for the potent allelopathy of *C. ericoides* (Tanrisever et al. 1987, Williamson et al. 1992, Fischer et al. 1994, Hewitt and Menges 2008) (Fig. 1). This process of light-activated allelopathy is unique to the Florida scrub (Fischer et al. 1994). *Ceratiola* is a monotypic genus and ceratiolin is not known to be produced by any other plant species. HCA is a phenolic acid and, while multiple examples of allelopathic action by phenolic acids are known (Blum 1996), HCA is a specific phytotoxin of native Florida grasses (Williamson et al. 1992, Fischer et al. 1994). The photoactivated allelopathic actions of ceratiolin and HCA have been studied in detail for their chemical properties and targeted effects on native plants (e.g., Tanrisever et al. 1987,

Jordan 1990, Williamson et al. 1992, Fischer et al. 1994, Hewitt and Menges 2008). However, the relationship between ceratiolin and the specialized insect herbivores of Florida rosemary remains to be examined.

Florida rosemary is well defended against herbivory and only a handful of insect herbivores, the majority specialists, are known (Sandoval-Mojica and Capinera 2011, Wheeler 2012). The aposematic two-striped walkingstick *Anisomorpha buprestoides* Stoll (Phasmatodea: Psuedophasmatidae) is the only generalist chewing herbivore reported to occasionally feed on *C. ericoides* (Conle et al. 2009). The few remaining generalist herbivores found on Florida rosemary are the piercing-sucking frugivores *Neopamera bilobata* Say and *Ozophora trinotata* Barber (Rhyparochromidae), and the scale insects *Neopulvinaria innumerabilis* Rathvon (Coccidae) and *Rhizaspidiotus dearnessi* Cockerell (Diaspididae) (Wheeler 2012, 2016). The specialist herbivores, too, are mostly piercing-sucking bugs, including *Alconeura bisagittata* Beamer (Cicadellidae), *Parthenicus weemsi* Henry (Miridae), *Diolcus chrysorrhoeus* Fabricius and *Homaemus proteus* Stål (Scutelleridae), both frugivores, *Thyanta custator custator* Fabricius (Pentatomidae), another frugivore, and *Keltonia balli* Knight (Miridae), a specialist feeder on staminate flowers (Wheeler 2009, 2012, 2016; Wheeler and Hicks 2012). There are only two known chewing insects that are specialized herbivores of *C. ericoides*: one is the moth *Nemoria outina* Ferguson (Geometridae) and the other is the focus of our study, the rosemary grasshopper, *Schistocerca ceratiola* Hubbell and Walker (Hubbell and Walker 1928, Deyrup and Eisner 1993).

The rosemary grasshopper is peculiar in that it exhibits strict monophagy on Florida rosemary, an exceptionally rare trait among grasshoppers. In fact, it is one of only two known strictly monophagous grasshopper species in North America, the other being *Bootettix argentatus* Bruner (Acrididae) associated with creosote bush, *Larrea tridentata* Coville (Zygophyllaceae) (Otte and Joern 1976, Chapman et al. 1988, Smith and Capinera 2005). The creosote ecosystem has some similarities to the Florida rosemary ecosystem, including a small community of herbivores with varying degrees of specialization for feeding on a host plant that produces formidable amounts of a unique secondary metabolite (Chapman et al. 1988). As such, the *B. argentatus*–creosote relationship is another system used to study the relationship between a specialized grasshopper and an abundant secondary plant metabolite in its diet (e.g., Chapman et al. 1988), but the rosemary grasshopper has an additional quality not found anywhere else.

The rosemary grasshopper is nocturnal, easily encountered at the tips of branches feeding on fresh leaves throughout the early night hours but seldom spotted during the day, an uncommon trait among grasshoppers (Hubbell and Walker 1928, Lamb and Justice 2005, C.C.G. and H.S., personal observation). Both nymphs and adults of
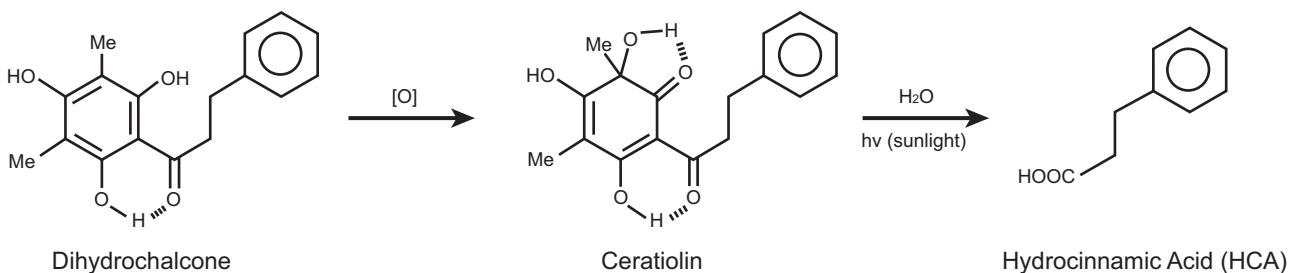

Fig. 1. The Ceratiolin Cascade. Adapted from Fischer et al. (1994).

S. ceratiola exhibit unique coloration patterns that render them cryptic on the different aerial tissues of rosemary bushes, likely a result of their inseparable relationship with their host plant. The adults are a more melanized morph, not black as much as brown and gray, camouflaged best against the leafless lower parts of the branches (Fig. 2a), while the nymphs are green with white to yellow markings, so they blend in among the many small needles as the older needles turn from green to yellow (Fig. 2b). Interestingly, the other specialist chewing herbivore of *C. ericoides*, the geometrid moth *N. outina*, has two larval color morphs to camouflage either in leaves or twigs, and *N. outina* is also a nocturnal feeder (Deyrup and Eisner 1993, Conle et al. 2009).

The rosemary grasshopper is an excellent system for investigating the physiological fate of ceratiolin within a specialist herbivore of Florida rosemary because its relatively large body facilitates the collection of greater quantities of regurgitant, hemolymph, and frass. The monophagy of *S. ceratiola* implies that it must ingest ceratiolin every time it feeds, but the fate of ceratiolin as it passes through the grasshopper's digestive tract is currently unknown.

Grasshoppers typically regurgitate their gut content when disturbed (Whitman 1990, Sword 2001), and it is likely that ceratiolin will be present in the regurgitant of *S. ceratiola* because it is the most abundant secondary plant compound in this grasshopper's diet (Jordan 1990). In a similar study (Fletcher et al. 2000), dihydrochalcones, the class of compounds to which ceratiolin belongs, were detected in frass when the grasshoppers fed on certain host plants, so it is likely that we will detect ceratiolin in the frass. HCA accounts for approximately half of the molecular weight of ceratiolin as a breakdown product and the breakdown products accounting for the other half have not yet been identified (Tanrisever et al. 1987, Fischer et al. 1994). The toxicity of HCA in grasshoppers has not been tested, to our knowledge.

We hypothesize that the breakdown products of ceratiolin represent potentially noxious compounds and *S. ceratiola* may exhibit nocturnal feeding behavior to avoid ingesting ceratiolin in direct sunlight where it readily decomposes. Associative learning in grasshoppers has been documented in response to the presence of noxious compounds in the hemocoel (Bernays and Chapman 2000). We set out to test if ceratiolin is restricted to the digestive tract of the grasshopper, or if it is transported into the hemocoel. To do this, we collected regurgitant, hemolymph, and frass from adult grasshoppers and performed qualitative analysis by liquid chromatography–mass spectrometry (LC–MS/MS). Additionally, we tested samples for the presence of HCA to determine whether ceratiolin degrades once inside the grasshopper.

Materials and Methods

Specimen Collection

We collected 10 adult grasshoppers on the night of 20 August 2017 in a rosemary bald located in Altoona, FL (29°07′33.2″N 81°34′37.2″W). We kept specimens in a cage with a 10:14 (L:D) h cycle at a constant 25°C. We provided cages with fresh rosemary branches from plants growing near the University of Central Florida main campus, placing the cut ends of the branches into a container with deionized water. We added water to the containers as needed to keep the rosemary hydrated and provided fresh water to the grasshoppers using a spray bottle to mist the rosemary branches. We collected frass, regurgitant, and hemolymph in that order from these 10 individuals on three consecutive days.

Frass Collection and Extraction

We collected frass by removing individuals from the rearing cage in the morning, rinsing them with deionized water for 10 s, and placing each of them in clean 50 ml Eppendorf tubes for 8 h of daytime. We rinsed grasshoppers with deionized water to remove ceratiolin and HCA which are likely present on the cuticle and might contaminate the sample. Sample collection began in the morning so that grasshoppers would be permitted the full extent of a normal nocturnal feeding schedule before sampling and would be returned to the cage to resume normal feeding afterward. Adult *S. ceratiola* do not feed during the day, so the frass in each sample was produced from the meal taken the night before isolation, and the isolation throughout the day did not disrupt their feeding schedule. We added 200 µl of 1:1 methanol:water (v/v) to each feces sample, vortexed briefly, and allowed to extract for 12 h in the dark at room temperature. The resulting yellow extract was passed through a 0.2 µm Teflon Luer lock filter and stored at 4°C until analysis.

Regurgitant Collection

To collect regurgitant, we removed grasshoppers from the cage in the afternoon, rinsed them in deionized water for 10 s, and placed them individually in 50 ml plastic tubes for 1 h prior to collection. By isolating grasshoppers in the afternoon, we would avoid bias for detecting
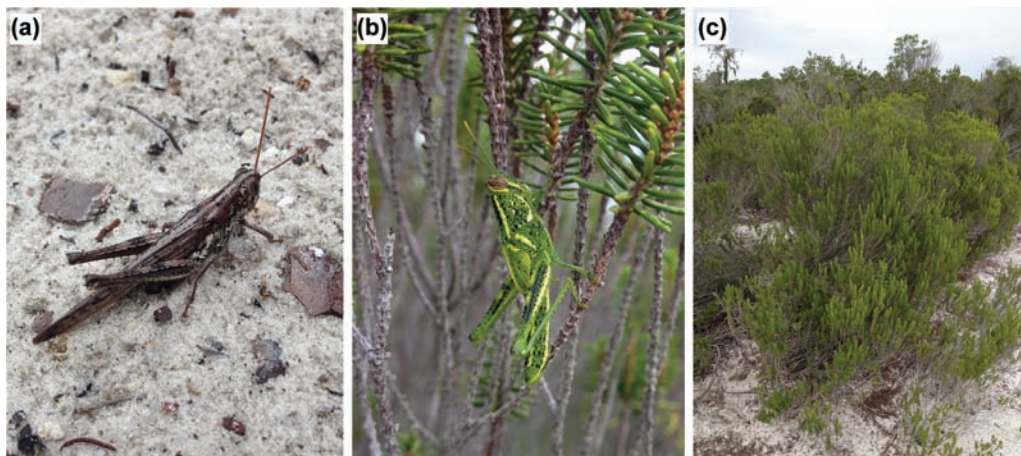

Fig. 2. (a) Adult female *S. ceratiola* ovipositing in the sand near its host plant. (b) A nymph with patterning for crypsis among the needle-like leaves. (c) A typical Florida rosemary bald.

ceratiolin or HCA in the regurgitant samples due to active feeding immediately before sampling because adult *S. ceratiola* feed only at night. We rinsed the grasshoppers in deionized water to minimize the amount of ceratiolin on the external surfaces of the mouthparts which might contaminate the regurgitant samples. The insects were given an hour in the tubes to dissipate water droplets. *S. ceratiola*, like many other species of grasshoppers, regurgitates when disturbed as one of their natural defense mechanisms. We collected regurgitant by grasping insects between the thumb and index finger, with the forelegs held dorsally to prevent interference, and pipetted directly from the mouth. We collected 5 µl of regurgitant from each grasshopper and diluted the sample to 50 µl in 1:1 methanol:water (v/v). We passed the diluted samples through a 0.2 µm Teflon Luer lock filter and stored the sample vials at 4°C until analysis.

Hemolymph Collection

For hemolymph collection, we handled the grasshoppers similar to the regurgitant collection with gloves and rinsing. Individuals were removed from the rearing cage in the afternoon, rinsed in deionized water for 10 s, and isolated for 1 h in clean centrifuge tubes before sampling. Hemolymph was directly pipetted through a small incision proximal to the coxa of a hind leg on the insect being sampled. We pipetted hemolymph directly through this incision. Hemolymph was collected on the final day of sampling because the hemolymph sampling process is the most damaging to the insects, compared with the frass and regurgitant sampling methods. We do not suspect that the regurgitant collection on the preceding day affected the presence or absence of ceratiolin or HCA in the hemolymph because the insects were allowed to feed normally throughout the night between sampling processes. We sampled the insects during daylight hours because it would best address our hypothesis that *S. ceratiola* might avoid sunlight if ceratiolin is present in the hemocoel. We collected 5 µl of hemolymph from each insect, diluted the sample to 50 µl in 1:1 methanol:water (v/v), filtered through a 0.2 µm Teflon Luer lock filter, and stored the sample vials at 4°C until analysis.

Isolation of Standard

Ceratiolin was isolated from the plant source for use as a standard. *C. ericoides* tissue was collected from a group of plants near the University of Central Florida in August 2017. Specifically, approximately 600 g of aerial plant parts were soaked in 6 liters of deionized water at room temperature for 24 h. We filtered the aqueous extract through cheese cloth and followed this with 20–25 µm (Grade 4) filter paper. Aqueous extracts were then purified on Acros Organics 17% C18 modified silica with 15% methanol in water to elute impurities and 1:1 methanol:dichloromethane (v/v) to elute the ceratiolin. The ceratiolin-containing fractions were then further purified by recrystallization in cold methanol following Tanrisever et al. (1987). Purified extracts were verified as ceratiolin by ^{13}C NMR in CD$_3$OD using a 500 MHz Varian VNMRS (Supp Fig. 1 [online only]). The purity of the ceratiolin extract was verified through a melting point determination with a value of 148–149°C observed (literature value 148–149°C) (Obara et al. 1989). HCA was purchased from Sigma-Aldrich (St. Louis, MO) for use as a standard.

Analytical Procedures and Qualitative Analysis

Ceratiolin was qualified by LC–MS/MS. Five microliters of extractions were injected into an Ascentis Express C-18 Column (3 cm × 2.1 mm, 2.7 µm) connected to a Sciex API3200 LC–MS/MS in (-) ESI mode. Chromatography was performed under a mobile gradient (time - %B) with water as solution A and acetonitrile as solution B at 0.275 ml/min: 2–5, 4–100, 6–100, 9–5%, 15-stop.

Samples were qualified by multiple reaction monitoring (MRM) of the m/z transition 301.1 > 283.0. HCA analysis was performed using the same chromatographic method and samples were qualified by MRM of the m/z transition 149.1 > 105.1.

Statistical Analysis

To determine whether the biological samples contained ceratiolin or HCA, we used a nonparametric approach to compare the mean detector response for the frass (n = 7), regurgitant (n = 10), and hemolymph (n = 10) samples to the mean detector response for blanks (n = 6). We used the integrated area under the extracted ion transition curve from each sample as the data points for our tests. We chose a nonparametric approach because the variance of the frass sample results for both ceratiolin and HCA were orders of magnitude greater than other sample groups (Supp Table 1 [online only]). We used a Kruskal–Wallis test followed by Dunn's posthoc using Holms' correction for multiple comparisons (Holms 1979). All statistical analyses were completed in R (R Core Team 2018).

Results

The isolated ceratiolin (C$_{17}$H$_{18}$O$_5$) standard displayed a peak in the MRM ion chromatogram for the m/z transition of 301.1 > 283.0 at about 8.5 min (Supp Fig. 2 [online only]). The HCA standard displayed a peak in the MRM ion chromatogram for the m/z transition of 149.1 > 105.1 at about 8.0 min. The results of the Kruskal–Wallis test indicated significant differences in ceratiolin content among groups (χ^2 = 14.772, df = 3, P = 0.002). We detected ceratiolin in the frass (P = 0.007) and regurgitant (P = 0.038), but not in the hemolymph (Fig. 3a). No significant differences in HCA content among groups were indicated by the Kruskal–Wallis test (Fig. 3b).

Discussion

This study investigated a possible connection between ceratiolin, a photoactivated plant compound, and the nocturnal behavior of the monophagous grasshopper that consumes it with every meal. We tested whether ceratiolin is restricted to gut or if it transports to the hemocoel. Our results indicate that ceratiolin is present in the regurgitant and frass, but not in the hemolymph. In contrast, HCA, the only known breakdown product of ceratiolin and the primary allelopathic agent, was not determined to be present in the regurgitant, hemolymph, or frass. Multiple frass samples produced relatively strong signals of HCA presence (Fig. 3b). We can infer from the abundance of ceratiolin in the frass samples that HCA should be present, because ceratiolin is known to readily decompose (Tanrisever et al. 1987); so perhaps with further replication, the presence of HCA in the frass could be confirmed. Our results do not support our hypothesis that the grasshopper may experience potentially noxious effects of ceratiolin breakdown in the hemocoel, but we continue to be intrigued by the coincidence of photoactivated ceratiolin and nocturnal specialized herbivores.

A study of Leichhardt's grasshopper, *Petasida ephippigera* White (Pyrgomorphidae), on three different host plants in the genus *Pityrodia*, showed that the dihydrochalcones from one host plant were present in the frass (Fletcher et al. 2000). Ceratiolin, a dihydrochalcone, though different than those found in *Pityrodia* due to its photoactivation, was detected in the frass in our study of *S. ceratiola*. Unlike the rosemary grasshopper, Leichhardt's grasshopper has intense aposematic coloration and is believed to sequester sesquiterpene glycosides from its host plants for defense (Fletcher et al. 2000). The fact that these two very different
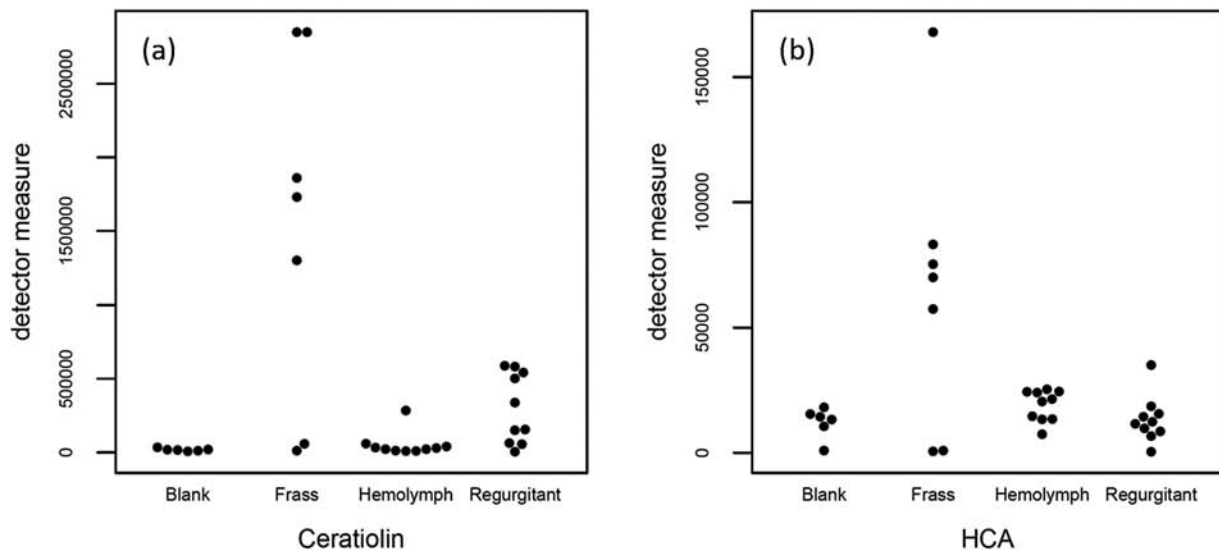
Fig. 3. Integrated peak areas (in counts per second) from liquid chromatography–tandem mass spectrometry of blanks, frass, hemolymph, and regurgitant samples. (a) Detection of ceratiolin. (b) Detection of hydrocinnamic acid (HCA).

grasshoppers are both efficient at filtering out and excreting dihydrochalcones from their host plants may be useful in examining metabolite detoxification across grasshopper clades.

One reason we are interested in studying the rosemary grasshopper is because it provides an opportunity to study the evolution of monophagy in a polyphagous clade. The ancestral grasshoppers are believed to have been polyphagous, given the high percentages of polyphagy in Acridoidea and the more basal Pyrgomorphoidea (Chapman and Sword 1997, Bernays and Chapman 2000). Within the family, there are subfamilies that prefer to feed on grasses such as Gomphocerinae, Acridinae, and Oedipodinae, which are believed to have radiated in the Cenonozic when the grasses radiated (Song et al. 2015). However, most other subfamilies prefer herbaceous plants, which appear to be a phylogenetically conserved trait within Cyrtacanthacridinae, to which *S. ceratiola* belongs. A recent phylogenetic study of *Schistocerca* suggests that the common ancestor of the genus must have been a swarming locust, which was likely to be a polyphagous insect (Song et al. 2017). *S. ceratiola* belongs to the Alutacea group, mostly oligophagous species that diversified in North America (Song 2004). Some species in this group show population-specific host plant specialization (Sword and Chapman 1994, Sword and Dopman 1999, Raszick and Song 2016), which suggests that perhaps ancestral *S. ceratiola* was predisposed to host plant specialization due to ecological trait conservatism. The Florida rosemary was a dominant plant that had a continuous distribution throughout the southeastern United States during the Pleistocene glacial maxima, and it expanded further due to drier climatic conditions (Trapnell et al. 2007). Because of the allelopathic property, it was probably the only available food source for the ancestral *S. ceratiola*, which could have promoted the evolution of monophagy. *Schistocerca* is a young genus, and the divergence of *S. ceratiola* is estimated to have taken place at the end of Pleistocene (Song et al. 2017), which fits well with the expansion of the Florida rosemary.

There is one other interesting pattern of evolution in this ecosystem that warrants further investigation. There are two known chewing insect herbivore specialists of Florida rosemary: *S. ceratiola* and *N. outina*. Coincidentally, both species have two color morphs to achieve crypsis on the plant, one morph is green to camouflage among the leaves and in both species the green morph is that of younger individuals. They have a brown morph to camouflage against the bare branches, and in *S. ceratiola* the

brown morph is that of adults, but in *N. outina* the brown morph is of the older larvae (Deyrup and Eisner 1993, Conle et al. 2009). In *N. outina*, the shift from green morph to brown morph is associated with a change in feeding behavior from younger to older leaves of *C. ericoides* (Conle et al. 2009).

In studies of these grasshoppers during 2013–2015, when *S. ceratiola* is maintained in a laboratory colony of both nymphs and adults, the adults will typically feed on the needles from the bottom up (from older leaves toward younger) while the nymphs typically feed from the top down (from younger leaves toward older) (C.C.G., personal observations). Perhaps there is a connection between the phytochemistry of the younger versus older leaves of *C. ericoides* that drives the evolution of two similar color polymorphisms in the two specialist chewing herbivores, and we hope this work inspires research therein.

Further research into a link between ceratiolin and the behavior of *S. ceratiola* should examine the levels of ceratiolin and HCA in regurgitant, hemolymph, and frass as a time course of 0, 3, 6, and 12 h postfeeding to investigate a possible temporal pattern in metabolite levels. Additionally, comparisons could be made of grasshoppers fed rosemary recently exposed to UV light (simulating day-time plant material) and rosemary not exposed to UV light (simulating night-time plant material). Rosemary balds are ecological islands with distinct communities of numerous endemic species that are increasingly threatened by human activity (Deyrup 1989, 1990; Lamb et al. 2006), and more chemical ecology studies of this system are needed before the opportunity is lost.

Supplementary Data

Supplementary data are available at *Annals of the Entomological Society of America* online.

Acknowledgments

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