J Chem Ecol (2008) 34:584–590
DOI 10.1007/s10886-008-9457-8

Developmental and Geographical Variation in the Chemical Defense of the Walkingstick Insect *Anisomorpha buprestoides*

Aaron T. Dossey · Spencer S. Walse · Arthur S. Edison

Received: 15 November 2007 / Revised: 11 February 2008 / Accepted: 19 February 2008 / Published online: 10 April 2008

Abstract *Anisomorpha buprestoides*, a walkingstick common in the southeastern United States, sprays chemicals that irritate and repel threatening insects, birds, or mammals. The active chemical in this substance was initially identified as a monoterpene dialdehyde. This compound can be present in several stereoisomeric forms, and subsequent studies have revealed that *A. buprestoides* produces at least three diastereomers: anisomorphal, dolichodial, and peruphasmal. However, no inquiry has been made to date into the geographical or developmental dependence of this variation. We report here that different populations of adult *A. buprestoides* spray either anisomorphal, or peruphasmal, or a mixture of the two stereoisomers. Additionally, offspring of a peruphasmal-producing population produced a variable mixture of anisomorphal and dolichodial but switched to peruphasmal upon reaching sexual maturity. This appears to be the first report of a developmentally regulated change in walkingstick insect chemical defense. Our results suggest a more complex role of these substances in the overall chemical ecology of walkingstick insects.

Keywords Insect · *Anisomorpha buprestoides* · Phasmatodea · Development · Chemical ecology · Monoterpene · Anisomorphal · Peruphasmal · Dolichodial

Electronic supplementary material The online version of this article (doi:10.1007/s10886-008-9457-8) contains supplementary material, which is available to authorized users.

A. T. Dossey · A. S. Edison (✉)
Department of Biochemistry & Molecular Biology,
University of Florida,
P.O. Box 100245, Gainesville, FL 32610-0245, USA
e-mail: art@mbi.ufl.edu

A. T. Dossey
e-mail: idpcwboy@ufl.edu

S. S. Walse
Center for Medical, Agricultural and Veterinary Entomology,
USDA-ARS,
Gainesville, FL 32604, USA

A. S. Edison
McKnight Brain Institute, University of Florida,
Gainesville, FL 32610, USA

A. S. Edison
National High Magnetic Field Laboratory, University of Florida,
Gainesville, USA

Present address:
S. S. Walse
Chemical Engineering, Yale University,
Mason Laboratory Room 307A, 9 Hillhouse Avenue,
New Haven, CT 06520-8286, USA

Introduction

Many living organisms rely on the use of chemical compounds for communication. Insects are particularly well-known for their chemical signaling and defense (Eisner et al. 2005). Many of the over 2,500 known species of walkingstick insects (Order Phasmatodea; Bedford 1978) produce noxious defensive secretions. However, the defensive chemistry of only a few species to date has been elucidated (Schneider 1934; Meinwald et al. 1962; Eisner 1965; Smith et al. 1979; Chow and Lin 1986; Ho and Chow 1993; Bouchard et al. 1997; Eisner et al. 1997; Schmeda-Hirschmann 2006; Dossey et al. 2006, 2007).

Defensive secretions from other types of insects are known to serve multiple functions (Blum 1996). For example, components of defensive secretions have been found to possess alarm pheromone activity in termites (Order Ispotera) and cockroaches (Order Blattodea) (Roisin

et al. 1990; Farine et al. 1997). Developmental changes in defensive secretion production have also been observed in insects such as true bugs (Order Hemiptera) (Blatt et al. 1998) and grasshoppers (Order Orthoptera) (Blum 1996). It has been postulated that walkingstick insect secretions could serve functions beyond warding off predators (Tilgner 2002), but so far, no studies have addressed this hypothesis.

We recently analyzed single defensive secretions from individual immature *Anisomorpha buprestoides* walkingsticks (Dossey et al. 2006) by using a novel 1-mm-high temperature superconducting NMR probe (Brey et al. 2006). This spray contained three monoterpene diastereomers: anisomorphal (Meinwald et al. 1962), dolichodial (Cavill and Hinterberger 1961; Cavill and Whitfield 1964; Cavill et al. 1976), and peruphasmal (Fig. 1a); glucose was also secreted with the monoterpenes (Dossey et al. 2006). Anisomorphal and dolichodial were produced in proportionally high concentrations compared with peruphasmal, which was present in only trace amounts. The ratios of anisomorphal and dolichodial varied from animal to animal and as a function of time (Dossey et al. 2006).

In two independent studies of *A. buprestoides* defensive spray, only anisomorphal was reported from *A. buprestoides* collected at Archbold Biological Research Station (Highlands Co., FL, USA) (Meinwald et al. 1962; Eisner et al. 1997). There were two major differences between the animals we worked with (Dossey et al. 2006) and those from the previous reports: (1) age/life stage and (2) geographical source of the animals from which spray was collected. Interestingly, *A. buprestoides* exists in various regionally specific color forms in Florida, USA (Hetrick

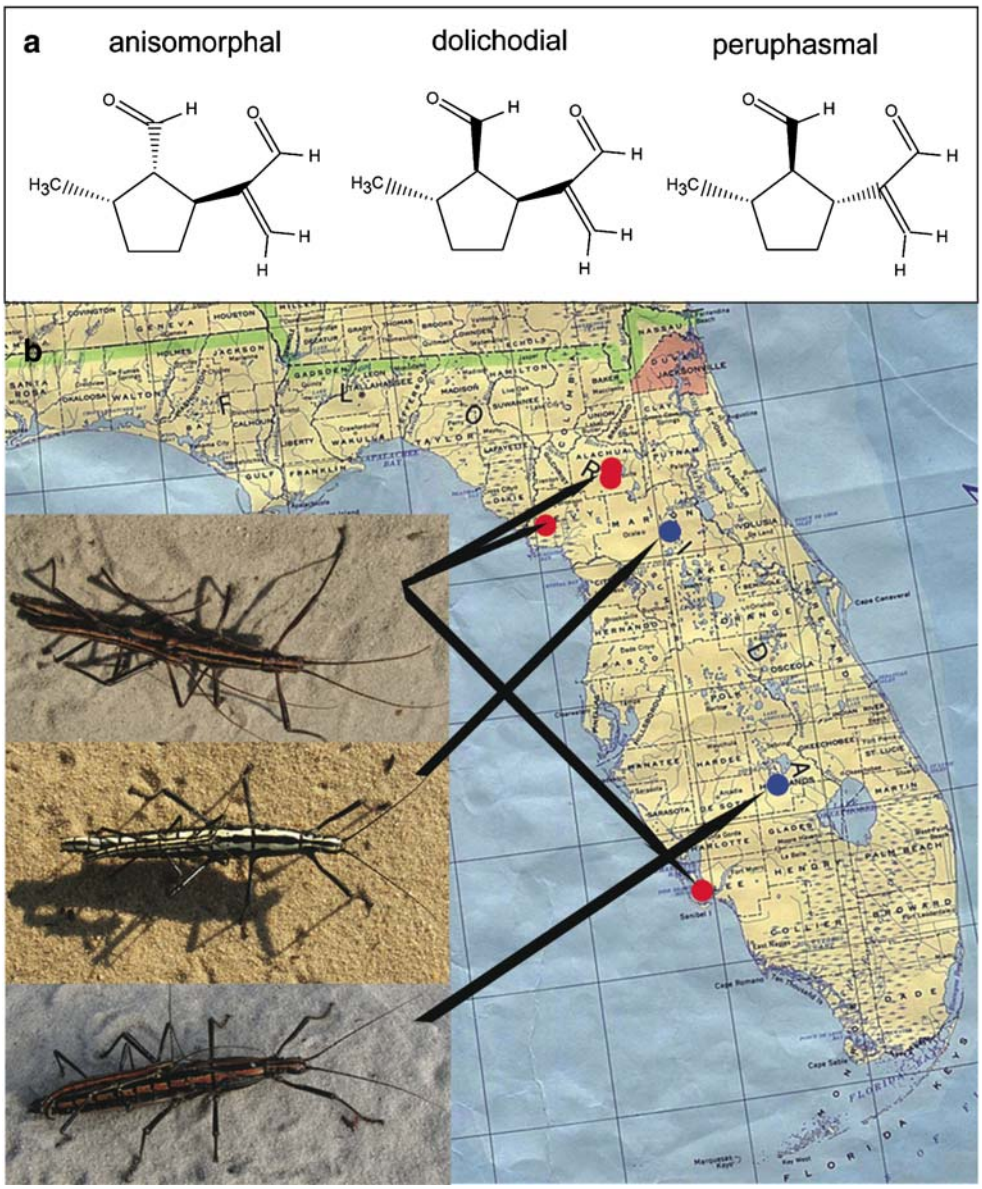

Fig. 1 Defensive spray variability in adult *A. buprestoides*. **a** The three monoterpene isomers produced by *A. buprestoides*. The relative configuration of dolichodial and anisomorphal were previously reported (Pagnoni et al. 1976). We have verified these studies and determined the relative configuration of peruphasmal (Wang et al., unpublished). **b** Geographic locations and photographs of color forms of *A. buprestoides* studied. The brown form (*top photo*) was found in two Gainesville locations (GNV and UFNA; 18 animals analyzed), GH (18 animals), and SI (three animals). The white-striped form (*middle photo*) was found in the ONF (15 animals). The orange-striped form (*bottom photo*) was found in AB (21 animals). The *colored dots* indicate collecting locations: *Blue dots* represent adult populations that only produced anisomorphal and *red dots* represent populations that produce peruphasmal or a mixture of peruphasmal and anisomorphal. One of the animals analyzed at Archbold produced about 25% dolichodial (Fig. 3), but that was the only significant amount of dolichodial found in the entire geographical study. No obvious dependence on gender of the animals was observed. Photographs by Aaron T. Dossey. The Florida map is courtesy of the University of Texas Libraries, The University of Texas at Austin

1949, Thomas 2001). The insects collected from Archbold for the current study all had a pair of orange dorsal stripes. Insects collected in Ocala National Forest (Florida, USA) were from a population with bright white dorsal stripes (Hetrick 1949). Aside from the stripes, the bodies of the Archbold and Ocala color forms are black. The bodies and dorsal stripes for *A. buprestoides* found at most other locations are usually brown with dark brown bodies and tan dorsal stripes. For simplicity, we refer to these color forms as the brown form (Fig. 1b, top photo), the white-striped form (Fig. 1b, middle photo), and the orange-striped form (Fig. 1b, bottom photo).

Here, we report both a developmental study and a geographical survey of the monoterpene dialdehydes used by *A. buprestoides* for defense. Additionally, we use ^{13}C labeling and ^{1}H NMR to demonstrate that *A. buprestoides* can synthesize its own defensive monoterpene dialdehyde from glucose. These results shed some light on the possible biological mechanisms behind the isomeric heterogeneity of defensive monoterpene dialdehyde production observed within this species.

Methods and Materials

Field Collections Individual secretions were collected in the field from sexually mature adult animals, as evidenced by the smaller males riding on the backs of larger females. The samples were frozen in the field and later analyzed by using NMR and gas chromatography-flame ionization detection (GC-FID). Each sample was an individual spray collected in a 1.5-ml glass vial held on top of the defensive gland while lightly agitating the insect. Samples were kept on ice and transported back to our lab for storage and analysis. The gender and location of each animal were recorded and are available in Supplementary Table S1. Global positioning satellite coordinates for collecting sites are as follows: Gainesville Airport "GNV": 29° 44′ 3″ N, 82° 16′ 27″ W; University of Florida Natural Area "UF-NA": 29° 38′ 1″ N, 82° 22′ 11″ W; Gulf Hammock "GH": 29° 14′ 46″ N, 82° 43′ 43″ W; Archbold Research Station "AB": 27° 10′ 50″ N, 81° 21′ 2″ W; Ocala National Forest "ONF": 29° 3′ 00″ N, 81° 38′ 50″ W; Sanibel Island "SI" two locations: 26° 27′ 5″ N, 82° 00′ 57″ W and 26° 27′ 37″ N, 82° 09′ 25″ W.

Developmental Study Eggs from the peruphasmal-producing GNV population of *A. buprestoides* were collected and reared in the laboratory. The hatchling animals were separated into their own plastic containers and reared as previously described (Dossey et al. 2006). Briefly, individuals were kept in separate containers, fed variegated privet (*Ligustrum sinense*), and raised from hatchling to adult. Defensive secretions were obtained as described for field collections of large animals or with a glass pipette placed directly over the defensive gland for very small animals. The secretions were extracted directly into methyl *tert*-butyl ether (MTBE) and analyzed by gas chromatography with mass spectrometric (GC-MS). The genders of the insects used in this study are available in Supplementary Table S2.

Isotopic Labeling An adult male *A. buprestoides* was fed 10–50 μl of 400 mM $^{13}C_6$ D-glucose (Cambridge Isotope Laboratories, Inc.) alternated every other day with variegated privet (*L. sinense*) for about 1 month. The glucose was given as a drop on the end of a blunt syringe needle and was readily accepted by the insect (Supplementary Video S1). Defensive secretions were collected periodically to monitor incorporation of ^{13}C into the defensive monoterpene. ^{13}C enrichment can easily be identified by large couplings between about 120 and 160 Hz (depending on the type of C–H bond) of the carbon-bound proton resonances in a standard ^{1}H 1D spectrum. This enrichment was quantified by using 1D ^{1}H NMR by dividing the sum of the integrals of the ^{13}C coupled "satellites" (e.g., pairs of coupled resonances) on each side of aldehyde ^{1}H resonances by the integral of the center ^{12}C-bound ^{1}H resonance.

Analytical Methods One-dimensional ^{1}H NMR spectra were collected as previously described (Dossey et al. 2006) on secretions dissolved directly with D_2O and subsequently analyzed with a custom built 1-mm HTS probe (Brey et al. 2006). GC-MS and flame ionization detection (GC-FID) were done as in (Dossey et al. 2006). Briefly, gas chromatography utilized He carrier gas (1.4 ml/min) and columns connected in series: a deactivated guard (L=8 cm, ID=0.53 mm), an HP-1MS retention-gap (L=2 m, ID=0.25 mm, df=0.25 mm), and a J&W DB-5 analytical (L=30 m, ID=0.25 mm, df=0.25 mm). Cool on-column or split-less injections (1 μl) were at 40°C and 200°C, respectively, and the oven program was isothermal at 40°C for 5 min, heated to 200°C at 11°C/min, and held 10 min, then heated to 250°C at 25°C/min and held 15 min. The FID was 260°C with N_2 make-up gas. Mass spectra were recorded with a Finnigan MAT Magnum® ion trap with 70-eV electron impact or isobutane chemical ionization over m/z 40–400; transfer-line and manifold temperatures were 240 and 220°C, respectively. Retention relative to tetradecane internal standard: peruphasmal, 0.81; dolichodial, 0.82; and anisomorphal, 0.83.

Results

Walkingstick Insects are Herbivores Thus, one explanation for chemical variability could be due to exogenous

synthesis of defensive compounds; if so, the different stereoisomers might be trivially explained as a consequence of diet. We note that dolichodial is also produced by ants (Cavill and Hinterberger 1961) and plants (Cavill and Hinterberger 1961; Pagnoni et al. 1976). To rule this out, we fed a few microliters of 400 mM $^{13}C_6$ D-glucose (see Supplementary Video S1) to a male walkingstick, alternating daily with food plant (privet—*Ligustum sinense*). We continued this feeding protocol for approximately 30 days and periodically analyzed the defensive secretions by NMR and mass spectrometry. Peruphasmal was enriched by 14%, 20%, and 25% in ^{13}C after 2, 3, and 4 weeks, respectively, clearly demonstrating that *A. buprestoides* can biosynthesize their own defensive monoterpene (Fig. 2).

Another explanation for chemical variability could be geographic location, so we sampled a total of seventy-five *A. buprestoides* from six different regions in Florida (Fig. 1b). As mentioned, *A. buprestoides* has at least three different color forms that are found in different geographical locations (Hetrick 1949; Thomas 2001). Figure 1b shows photographs of each color form, geographical collecting locations, and a summary of the defensive spray composition from insects in each location. Quantitative results are shown in Fig. 3. The white-striped (Hetrick 1949) and orange-striped forms collected in the Ocala National Forest and Archbold Research Station, respectively, secreted only anisomorphal, consistent with the original studies (Meinwald et al. 1962; Eisner et al. 1997). The brown forms, collected in Gainesville, Gulf Hammock and Sanibel Island, were more heterogeneous, producing only anisomorphal (28 animals), only peruphasmal (five animals), or a mixture of anisomorphal and peruphasmal (six animals). With one exception (Fig. 3), no adult *A. buprestoides* produced more than a trace amount of dolichodial.

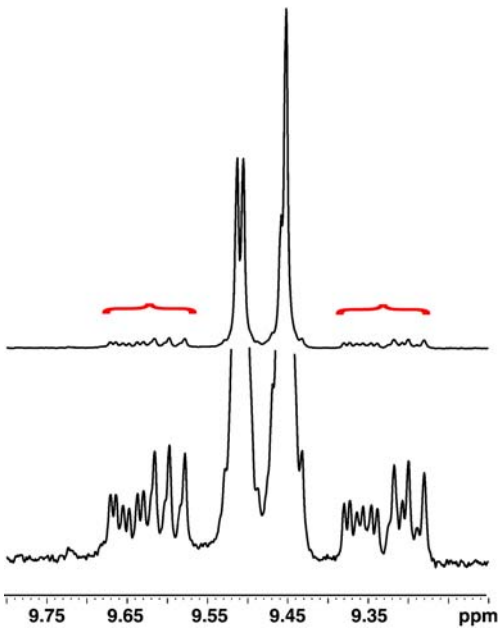

Fig. 2 Isotopic labeling of *A. buprestoides*. Animals were fed 10–50 µl of 400 mM uniformly labeled ^{13}C glucose alternated with plants for 15–30 days. This sample was collected on day 17, and 14% of the carbons in peruphasmal were labeled with ^{13}C, demonstrating that the insects can synthesize their own venom. The ^{13}C incorporation increased to 25% at 30 days (not shown). The spectrum shown is an expansion of the aldehyde region; the *top* shows the full intensity of the peaks, and the *bottom* is the same spectrum vertically expanded to show details of the ^{13}C satellites, indicated by *brackets*. The satellite resonances result from large one-bond ^{13}C–^{1}H scalar coupling, and the amount of ^{13}C incorporation can be easily determined from the ratio of the sum of the integrals of the satellite resonances to the integral of the central, ^{12}C-bound, resonances. A video showing the feeding is available as Supplementary Material, video S1

In our previous study, we found significant but variable amounts of dolichodial in immature *A. buprestoides* (Dossey et al. 2006). Because we found almost no dolichodial in the adult populations (Fig. 3), we investigated the role of development in the chemical composition of defensive secretions. Fourteen animals (ten males, three females, and one, which died before gender could be determined) were reared from hatchlings to adults as described in "Methods and Materials". These were offspring of adults collected from a peruphasmal-producing Gainesville population (GNV). Defensive secretions were collected and analyzed by GC-MS over a period of about 3 months (Fig. 4). Immature hatchlings (Fig. 4a) produced significant but variable amounts of dolichodial and anisomorphal but only trace amounts of peruphasmal, consistent with our previous findings (Dossey et al. 2006). We observed a consistent trend within each individual animal of increasing amounts of dolichodial after about 2 months of development (see yellow and orange circles in Fig. 4b and Supplementary Table S2 with a complete table of results). To our surprise, upon reaching sexual maturity, all animals stopped producing dolichodial and anisomorphal and started secreting only peruphasmal. Additionally, hatchlings reared in identical conditions but from another brown form population (Gulf Hammock, FL, USA) produced anisomorphal as adults (data not shown). This verified that peruphasmal was not the only isomer produced by adults reared under these conditions. Interestingly, these developmental changes in isomer production corresponded to the same sub-adult developmental stage when the males would normally mount the backs of females, the standard behavior for *A. buprestoides*.

Discussion

The results illustrate several aspects of the chemical defense system utilized by *A. buprestoides*. First, it is able to synthesize its own defensive monoterpene *de novo* from D-glucose. We hypothesize that the variability in the defensive

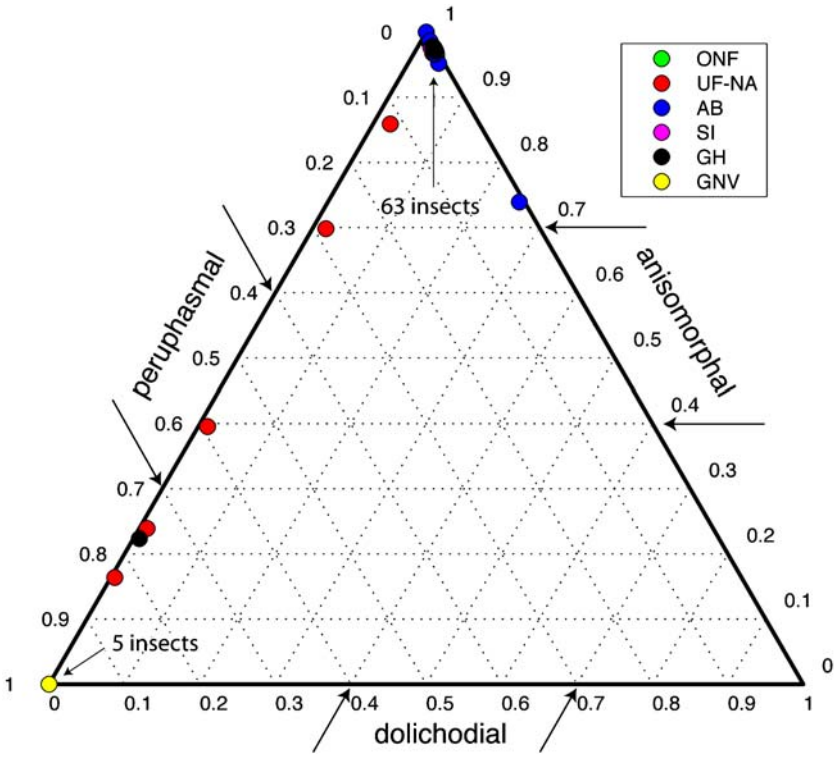

Fig. 3 Field milkings of individual adult *A. buprestoides*. Single defensive secretions were collected from animals in six different geographic locations in Florida (see Fig. 1b for abbreviations). The samples were frozen and analyzed by GC-FID and/or NMR as described in "Methods and Materials", and the total amount of anisomorphal, peruphasmal, and dolichodial for each animal was normalized to 1. The *arrows* on ternary plot axes indicate the direction that each component is plotted (e.g., anisomorphal is *horizontal*). With the exception of one sample with 25% dolichodial from AB, all other samples contained predominantly anisomorphal (63 insects), peruphasmal (five insects), or a mixture of anisomorphal and peruphasmal (six insects)

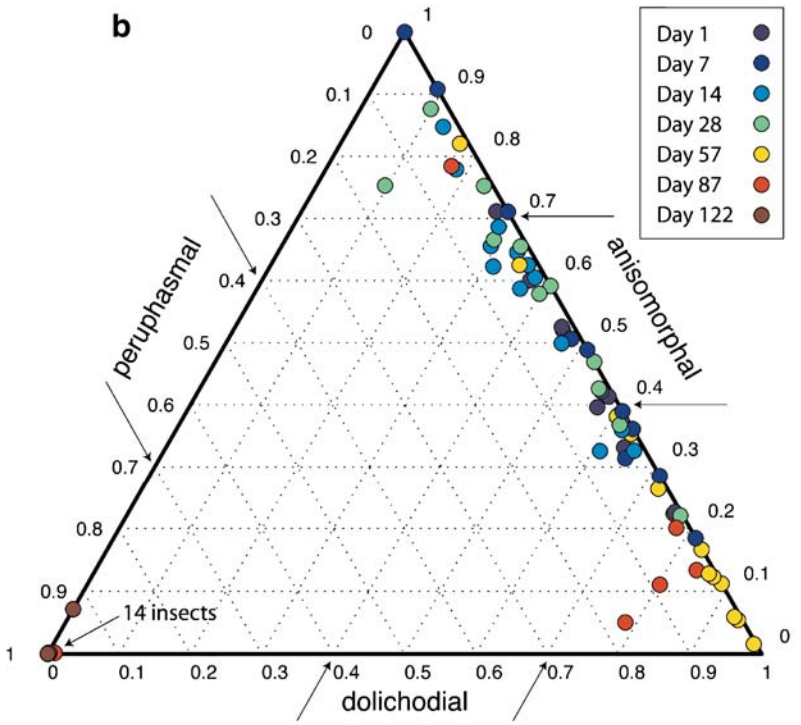

Fig. 4 *A. buprestoides* defensive secretions as a function of development. Fourteen animals (ten males, three females, and one undetermined) from a peruphasmal-producing GNV population (Figs. 1b and 3) were raised from hatchling to adult in individual containers. Individual secretions were collected from 1- to 3-day-old hatchlings (**a**) and at subsequent intervals ranging from 1 to 5 weeks, and secretions were analyzed by GC-MS. **b** Ternary plot of normalized anisomorphal, peruphasmal, and dolichodial concentrations, as described in Fig. 3. The data from *Day 1* were from 1- to 3-day-old hatchlings. By *Day 122*, all animals had reached sexual maturity. Photograph by Aaron T. Dossey

secretion composition likely results from variation in insect biosynthetic enzyme expression, primary sequence, or both. Such a system could evolve independently of food plant selection. However, further experiments are needed to determine whether enzymes or other non-protein mechanisms such as acid/base catalysis are responsible for the variability. Second, different populations of *A. buprestoides* produce different ratios of monoterpene dialdehydes in their defensive secretions, which also suggests an underlying genetic mechanism. Population dependent variability also could be the result of group isolation and, considering drastic differences between different color forms, the early stages of speciation. Since these insects do not fly, gene flow across their geographic range is probably slow, and certain populations are likely to become genetically isolated over time. However, a complete population genetic study of this species has not been pursued and is beyond the scope of the current study.

Finally, the composition of the monoterpene dialdehydes in *A. buprestoides* defensive spray is dependent on developmental life stage, showing both a trend to increased levels of dolichodial at about 2 months and an abrupt transition to ~100% peruphasmal by about 4 months (Fig. 4b). In the geographic study, we also found that adult *A. buprestoides* may sometimes produce anisomorphal or a combination of anisomorphal and peruphasmal, depending on which population they come from. In contrast, although dolichodial can be a major component of the spray before animals reach sexual maturity (Fig. 4b; Dossey et al. 2006), it is present only in trace amounts in adults (Fig. 3). One possible explanation for these observations is adaptive response to different predatory environments. For example, it is possible that young *A. buprestoides* encounter smaller invertebrate predators such as other insects or spiders. These may be more sensitive to dolichodial. Adult *A. buprestoides* are probably more vulnerable to attack by large vertebrate animals such as birds or mammals. It is possible that these animals are less sensitive to dolichodial and more sensitive to anisomorphal and peruphasmal. The developmental changes in *A. buprestoides* defensive spray chemistry also suggest their possible use as pheromones. *A. buprestoides* have an unusual behavior among insects: Males begin to copulate with the females before the females are fully grown, with one or two molts remaining. It is, therefore, possible that *A. buprestoides* defensive compounds also function as mating pheromones to regulate this behavior. In fact, many walkingstick species do not produce any secretion from their thoracic defense glands or produce secretions that are not irritating, at least to humans (Tilgner 2002). However, these hypotheses have yet to be tested. Overall, the findings of this study emphasize the value of extensively sampling natural products over both development and geographic range at individual organism resolution in order to more fully understand underlying chemical biodiversity and ecology.

Acknowledgments We thank Drs. W. Brey (NHMFL), R. Withers (Varian), R. Nast (Varian), and Bruker for the 1-mm HTS NMR probe. J. Rocca (AMRIS) provided help with NMR, and O. Conle (Bolsterlang, Germany) provided advice on walkingsticks. Dr. P. Teal (USDA) provided support and laboratory resources. Dr. Omjoy Ganesh (UF) provided technical help with making ternary diagrams. Drs. P. Sternberg (Caltech) and S. Benner (The Westheimer Institute of Science and Technology) provided helpful comments on the text. Funding was from the Human Frontiers Science Program and the NSF funded National High Magnetic Field Laboratory. The authors declare no competing financial interests.

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