

Adults and Nymphs Do Not Smell the Same: The Different Defensive Compounds of the Giant Mesquite Bug (*Thasus neocalifornicus*: Coreidae)

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Abstract Heteropteran insects often protect themselves from predators with noxious or toxic compounds, especially when these insects occur in aggregations. The predators of heteropteran insects change from small insect predators to large avian predators over time. Thus, a chemical that is deterrent to one type of predator at one point in time may not be deterrent to another type of predator at another point in time. Additionally, these predator deterrent compounds may be used for other functions such as alarm signaling to other conspecifics. Defensive secretion compounds from the adult and the nymph giant mesquite bug (*Thasus neocalifornicus*: Coreidae) were isolated and identified by gas chromatography–mass spectrometry and NMR. The predominant compounds isolated from the nymph mesquite bugs during a simulated predator encounter were (*E*)-2-hexenal and 4-oxo-(*E*)-2-hexenal. In adults, the major compounds released during a simulated predator encounter were hexyl acetate, hexanal, and hexanol. Results from predator bioassays suggest the nymph compounds are more effective at deterring an insect predator than the adult compounds. By using behavioral bioassays, we determined the role of each individual compound in signaling to other mesquite bugs. The presence of the nymph secretion near a usually compact nymph aggregation caused nymph mesquite bugs to disperse but did not affect adults. Conversely, the presence of the adult secretion caused the usually loose

adult aggregation to disperse, but it did not affect nymph aggregation. The compounds that elicited nymph behavioral responses were (*E*)-2-hexenal and 4-oxo-(*E*)-2-hexenal, while those that elicited adult behavioral responses were hexyl acetate and hexanal. The differences between the chemical composition of nymph and adult defensive secretions and alarm behavior are possibly due to differences in predator guilds.

Keywords Aggregation · Carbonyl defense · Coreidae · Defensive chemistry · Warning coloration

Introduction

Many species of warning colored insects have evolved chemical defenses that deter predatory attack and increase insect survival (Blum 1981; Eisner et al. 2007). However, the identification of which chemical is important in conferring functional benefits is difficult since insects often contain many compounds, and chemical mixtures may change in both space and time. The predators' behavioral response also varies with the identity of the predator (Eisner 1970; Eisner et al. 2007). Complications arise when compounds have multiple functions such as deterring predators and alerting conspecifics to predator attack (Eisner 1970; Eisner et al. 2007). Despite these complications and difficulties, however, an understanding of how these compounds function and to whom they are signaling is imperative in understanding adaptive significance and evolutionary history.

Heteropteran insects are known for their chemical defenses and gregarious behavior (Aldrich 1988; Millar 2005). Typically, the defensive secretions are mixtures of aliphatic compounds, most with carbon chains of C₄, C₆,

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and C_8 (Aldrich and Yonke 1975; Aldrich 1988; Millar 2005). These compounds are thought to be more effective at deterring arthropod predators than birds or mammals (Eisner 1970). They may function not only as toxins and repellents to predators, but also as alarm pheromones to conspecifics, thereby alerting and dispersing threatened aggregations (Calam and Youdeowei 1968, Aldrich and Blum 1978; Blum 1981; Leal et al. 1994). However, it is often unclear which chemicals are defensive against predators, which elicit a dispersal behavior among conspecifics, and whether those chemical identities change through different life stages of insects.

The genus *Thasus* consists of neotropical and neosubtropical coreid bugs that range from the American southwest to northern South America. This genus contains some of the largest species of Heteroptera with some reaching over 50 mm in length (Brailovsky et al. 1994). Giant mesquite bugs (*Thasus neocalifornicus*: Coreidae) are among the largest heteropterans of the desert southwest USA. These insects appear to have different visual defensive strategies between the nymph and adult life stage. Adults are large (~40 mm), cryptic, and occur in small, loose aggregations, while nymphs are smaller (~5–30 mm), highly conspicuous, and occur in larger, compact aggregations (Olsen 2004). Both adults and nymphs occur simultaneously on the same host, and both life stages emit a visible secretion when disturbed. They are specialists on mesquite tress (*Prosopis* spp.: Fabaceae), and are frequently seen in southeastern Arizona, especially around Tucson

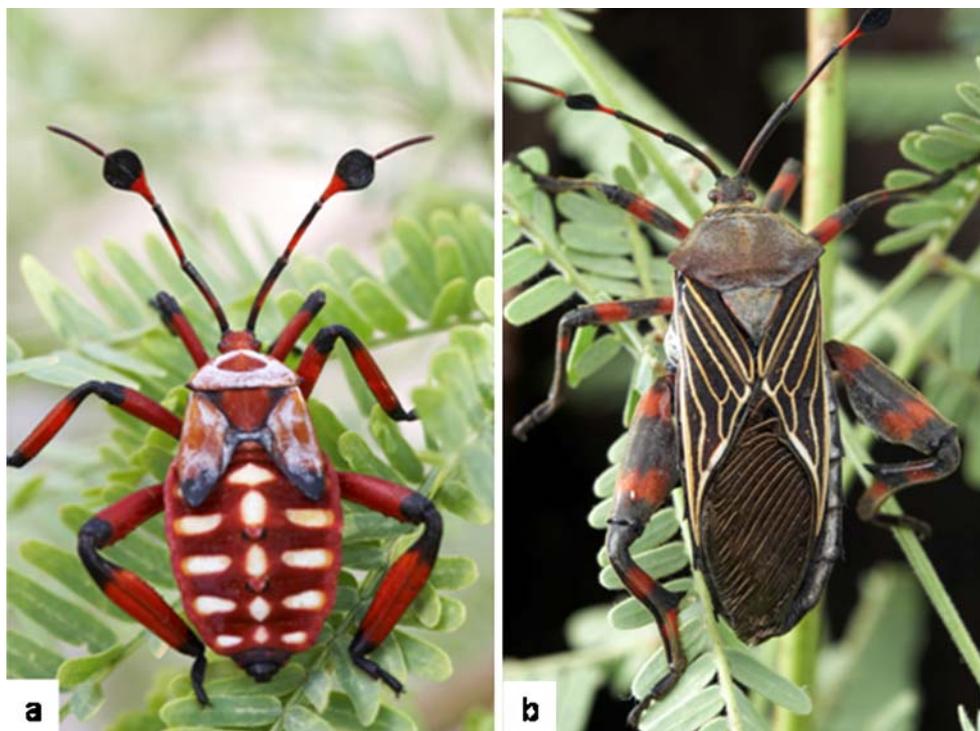
(Forbes and Schaefer 2003). Nymphs are seen more often because of their conspicuous feeding aggregations on the young petioles or seed pods of mesquite (Olsen 2004). They are vividly colored red and white (Fig. 1). Adult giant mesquite bugs are colored dark olive with dark red and black legs (Fig. 1). Their size probably protects them from many insect predators, but they are attacked by lizards and birds (Olsen 2004). Both nymphs and adults emit a foul-smelling odor when disturbed, and they appear to disperse as observed in other closely related species (Aldrich and Blum 1978). Because of their different visual defensive strategies and potential predators, we investigated whether nymph and adult giant mesquite bugs contained different defensive compounds and whether those compounds elicited different behavioral responses in nymphs and adults.

By using gas chromatography–mass spectrometry (GC–MS) and ^1H and ^{13}C NMR, we investigated whether nymph and adult giant mesquite bugs emit different chemical compounds when disturbed. Once we identified the compounds, we used behavioral assays to determine: (1) if nymph chemicals were better at deterring insect predators than adult chemicals; and (2) if nymph and adult chemicals elicited similar or different escape behaviors in nymphs and adults.

Methods and Materials

Insect Collection Adults and third instar nymphs were collected off host plant, Arizona mesquite trees (*Prosopis*

Fig. 1 Photo of **a** nymph and **b** adult giant mesquite bug, *Thasus neocalifornicus*, courtesy of Alex Wild



velutina: Fabaceae) on June 30th, 2007 from two locations in Tucson, Pima County, AZ, USA: The University of Arizona campus (32.23°N, 110.95°W) and the Tucson Botanical Gardens (32.24°N, 110.91°W). Giant mesquite bugs were kept in the lab and fed Arizona mesquite leaves and pods for the period of experimentation.

Preparation of Extracts To collect the secretion emitted during a predation event, we pinned either an adult or a nymph (instar 3 or 4) onto a glass plate with forceps and collected the secretion from the abdomen on filter paper. The filter paper was extracted in 1 ml hexane for 5 min, and the extract was analyzed by GC and GC–MS.

To confirm our results, we also extracted whole insects. Nymphs (instar 4 or 5) were dipped into hexane (5 ml) for 3–5 min, and the extract was analyzed by GC and GC–MS. Adults were extracted with 10 ml hexane and analyzed in the same way. For identification of 4-oxo-(*E*)-2-hexenal, eight nymphs were extracted with hexane for 3 h. The extract was concentrated and then applied onto a SiO₂ column (300 mg), and successively eluted with 3 ml each of hexane, 3, 4, 10, and 50% ethyl acetate in hexane. 4-Oxo-(*E*)-2-hexenal (2.4 mg) was eluted purely in the 10% ethyl acetate fraction.

Chemical Analyses and Identification Gas chromatography (GC) analysis was performed with an Agilent 6890N GC with a flame ionization detector, by using an HP-5MS capillary column (Agilent Technologies, 30 m×0.25 mm i.d., 0.25 μm film thickness) with helium carrier gas at 2.0 ml/min in splitless mode. The oven temperature was programmed to change from 50°C (3-min holding) to 300°C at 10°C/min and then held for 5 min. The injector temperature was maintained at 200°C, and the detector temperature at 300°C. The relative percentages of each component were calculated based on the peak area by flame ionization detector (FID).

GC–Mass Spectrometry (GC–MS) analysis was performed with the Agilent 6890 N GC linked to an Agilent 5975B operated at 70 eV with an HP-5MS capillary column (Agilent Technologies, 30 m×0.32 mm i.d., 0.25 μm in film thickness) under the same analytical conditions described above, except the velocity of helium carrier gas was 1.2 ml/min.

All compounds except for 4-oxo-(*E*)-2-hexenal were identified by comparing their GC retention times and mass spectra with those of authentic standards. (*E*)-2-Hexenal, hexyl acetate, hexanol, and hexanal standards were purchased from Sigma Chemical Co. (USA). 4-Oxo-(*E*)-2-hexenal was synthesized by a one-step reaction described in Moreira and Millar (2005) and verified by GC–MS. The structure of the 4-oxo-(*E*)-2-hexenal was confirmed by ¹H and ¹³C NMR spectra by using the purified natural

compound. ¹H and ¹³C NMR spectra were acquired on a Bruker DRX-500 spectrometer (¹H at 500 MHz and ¹³C at 125 MHz) with a Bruker Dual (¹³C/¹H) 5-mm probe at a sample temperature of 25°C in a CDCl₃ solution with tetramethylsilane (TMS) as an internal standard.

Mesquite Bug Behavioral Assays Mesquite bugs were organized into groups of five individuals and placed into separate arenas measuring 25 by 35 cm. Each group was composed of either third instar nymphs or adults (in total: three groups per life stage). Within each group, individuals were labeled one through five. During each trial, a single focal individual was observed for behavioral changes. The identity of the focal individual rotated among replicates. Volatiles were introduced into the arena via a piece of cotton with 3 μl of compound. The treatment compounds were distilled water (control treatment), nymph secretion collected from the abdomen, adult secretion collected from the abdomen, 4-oxo-(*E*)-2-hexenal, (*E*)-2-hexenal, hexyl acetate, hexanol, hexanal, 4-oxo-(*E*)-2-hexenal+(*E*)-2-hexenal, and hexyl acetate+hexanol+hexanal (*N*=15 observations/treatment). We measured the change in distance (cm) to the volatile source, change in number of neighbors in 5-cm radius, and change in distance to the nearest bug neighbor (0–43 cm) before and 2 min after the volatile(s) was introduced into the arena. All data were evaluated for normality and homoscedasticity. Count data were square root transformed to normalize. Data were analyzed with one way ANOVA by comparing control response to treatment response with JMP-In 5.0 (SAS Institute 2002). Bonferroni alpha level corrections were made to reduce type I errors.

Insect Predator Behavioral Assays California mantids, (*Stagmomantis californica*: Mantidae), were hand reared from oothecae collected on the University of Arizona campus. They were tested during their 4th instar and averaged 40 mm in length. A mantid was fed a maintenance diet of two crickets per day. In addition, for the first experiment, each mantid was fed a cricket the 1st day and a giant mesquite bug nymph the next (*N*=5). Cricket and nymph were paired based on weight. We recorded whether the mantid oriented, attacked, and ate each prey type. For the second experiment, crickets were treated with 2 μl of either distilled water (control treatment), or nymph secretion, adult secretion, 4-oxo-(*E*)-2-hexenal, (*E*)-2-hexenal, hexyl acetate, hexanol, or hexanal. These crickets were fed to another set of 4th instar mantids. We recorded whether the mantid oriented, attacked, and ate each prey type. Each mantid experienced one water treatment and one other treatment (*N*=5 per treatment, *N*=35 across all treatments). All data were evaluated for normality and homoscedasticity. Data were analyzed with a log-linear model by using JMP-In 5.0 (SAS Institute, 2002).

Results

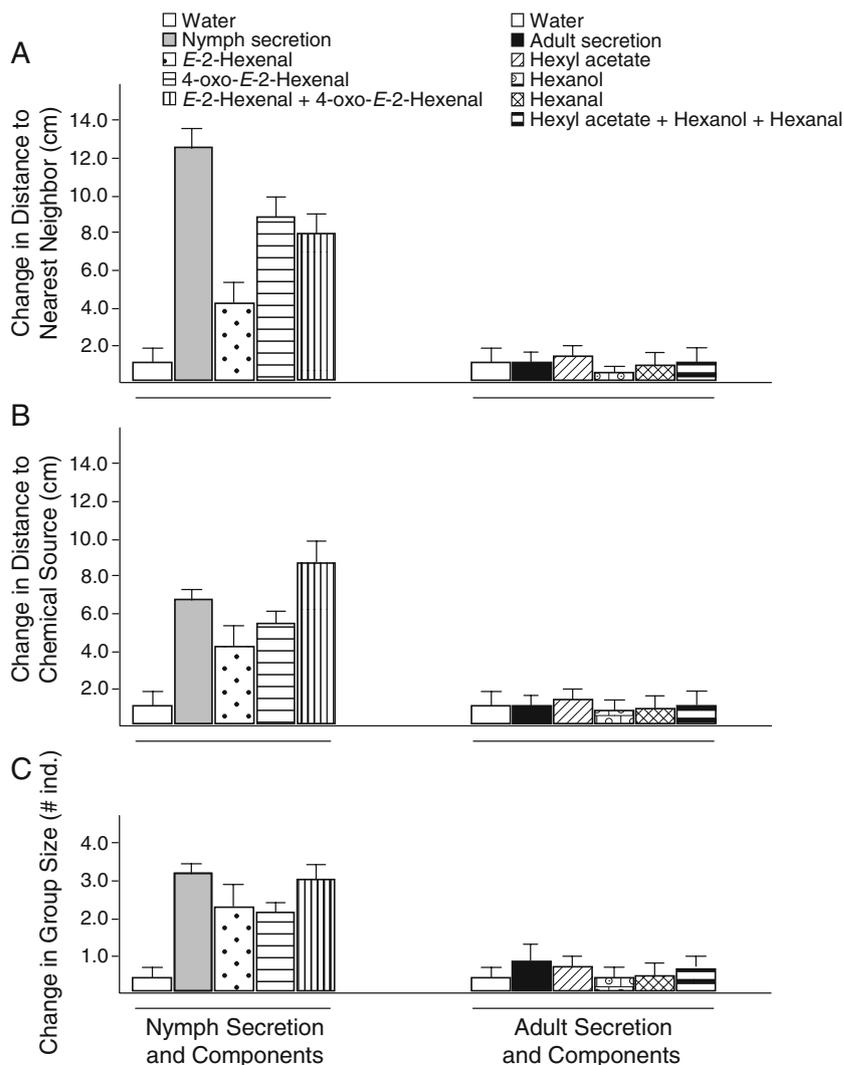
Mesquite Bug Chemical Profiles Giant mesquite bug nymphs emitted (*E*)-2-hexenal and 4-oxo-(*E*)-2-hexenal, while adults emitted hexyl acetate, hexanal, and hexanol when disturbed. The two life stages had no compounds in common. The same pattern of compounds was also observed in the whole body extraction. Female and male adults differed slightly in the relative amounts of each compound, but these differences were not significant.

The mass and NMR spectra of purified 4-oxo-(*E*)-2-hexenal are summarized as follows: MS *m/z* (%): 112 (*M*⁺, 15), 84 (14), 83 (100), 57 (13), 55 (49); ¹H NMR (CDCl₃): δ 9.77 (1H, d, *J*=7.2 Hz), 6.86 (1H, d, *J*=16.3 Hz), 6.77 (1H, dd, *J*=16.3, 7.2 Hz), 2.72 (2H, q, *J*=7.2 Hz), 1.15 (3H, t, *J*=7.2 Hz); and ¹³C NMR (CDCl₃): δ 200.31, 193.34, 144.69, 137.29, 34.52, 7.54. The total amount of 4-oxo-(*E*)-2-hexenal was 2.4 mg from eight nymphs for NMR analysis. The NMR spectra of the

purified compounds were consistent with published spectra (Moreira and Millar 2005).

Mesquite Bug Behavioral Responses There was no effect of arena on mesquite bug behavior ($F_{1,5}=1.22$, $P=0.23$). Therefore, data were pooled across arena groups within a life stage. As compared to distilled water, nymph giant mesquite bugs disperse more in the presence of their own defensive compounds. Response by nymphs to adult secretion or any of the individual adult compounds did not differ from their response to water (Fig. 2; $P>0.05$). The presence of nymph secretion caused nymphs to travel farther away from the chemical source ($F_{1,14}=5.33$, $P=0.012$), to have fewer nymphs in a 5 cm radius ($F_{1,14}=6.52$, $P<0.01$), and to be farther away from the nearest giant mesquite bug ($F_{1,14}=9.74$, $P<0.01$). (*E*)-2-hexenal and 4-oxo-(*E*)-2-hexenal alone elicited bugs to travel away from the source ($F_{1,14}=3.73$, $P=0.012$ and $F_{1,14}=12.34$, $P<0.01$, respectively), but a mixture of these two compounds was

Fig. 2 Nymph behavioral responses to water (the control treatment), nymph secretion, adult secretion, and various authentic compounds from each secretion. We measured several variables: **a** change in distance to nearest neighbor, **b** change in distance to chemical source, and **c** change in aggregation group size after the treatment compound had been in the arena for 2 min. Means±SE shown ($N=15$ bugs per treatment)



almost as effective as the actual nymph secretion ($F_{1,14}=8.63, P<0.01$). The same pattern was found also in both change in distance to nearest neighbor ($F_{1,14}=7.90, P<0.01$) and change in group size ($F_{1,14}=8.84, P<0.01$).

Compared to distilled water, adult giant mesquite bugs also dispersed more in the presence of their own defensive compounds (Fig. 3). The presence of the adult secretion resulted in fewer adults nymphs in a 5 cm radius ($F_{1,14}=5.60, P=0.01$) and farther away from the nearest adult bug ($F_{1,14}=4.22, P=0.03$). Adults also tended on average to travel farther away from the adult secretion source, but the difference was not significant after the Bonferroni correction ($F_{1,14}=2.03, P>0.05$). Hexyl acetate and hexanal alone elicited dispersal behavioral responses ($P<0.02$ for all comparisons), but hexanol did not ($P>0.05$ for all comparisons). A mixture of all three compounds was almost as effective as the actual adult secretion in changing the distance to source ($F_{1,14}=3.04, P=0.05$) and a change in

group size ($F_{1,14}=6.29, P=0.01$). None of the behavioral responses by adults to nymph secretion or to any of the individual nymph compounds differed from the response to distilled water, the control treatment ($P>0.05$ for all comparisons).

Insect Predator Responses Compared to crickets, nymph mesquite bugs were not as palatable to mantids (Fig. 4). Untreated crickets were always oriented on, attacked, and eaten by mantids, while giant mesquite bug nymphs were oriented and attacked by mantids, but often released before consumption ($F_{1,9}=8.46, P=0.004$). Crickets treated with nymph secretions were more unpalatable to mantid predators than crickets treated with adult secretions ($F_{1,9}=12.47, P=0.003$). This effect was best explained by the presence of 4-oxo-(*E*)-2-hexenal ($F_{1,9}=13.86, P=0.002$). This compound caused four of the five mantids to die after attacking those crickets even though the crickets were not consumed.

Fig. 3 Adult behavioral responses to water (the control treatment), nymph secretion, adult secretion, and various authentic compounds from each secretion. We measured several variables: **a** change in distance to nearest neighbor, **b** change in distance to chemical source, and **c** change in aggregation group size after the treatment compound had been in the arena for 2 min. Means+SE shown ($N=15$ bugs per treatment)

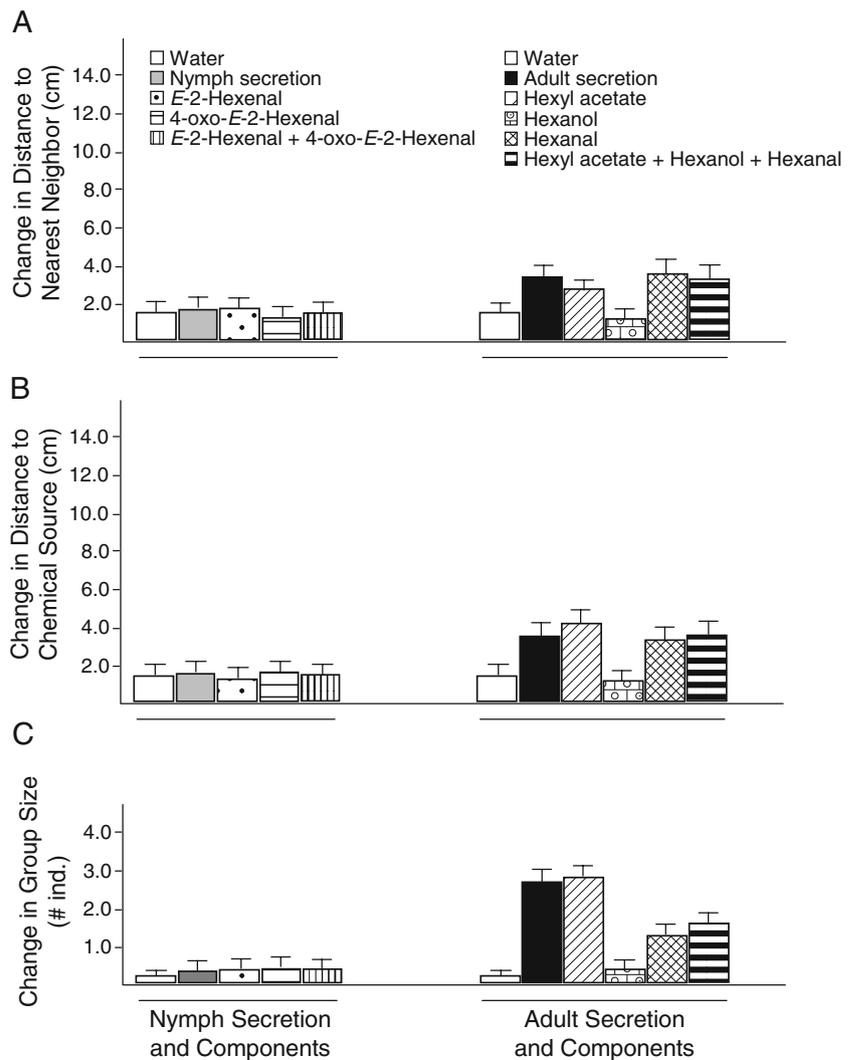
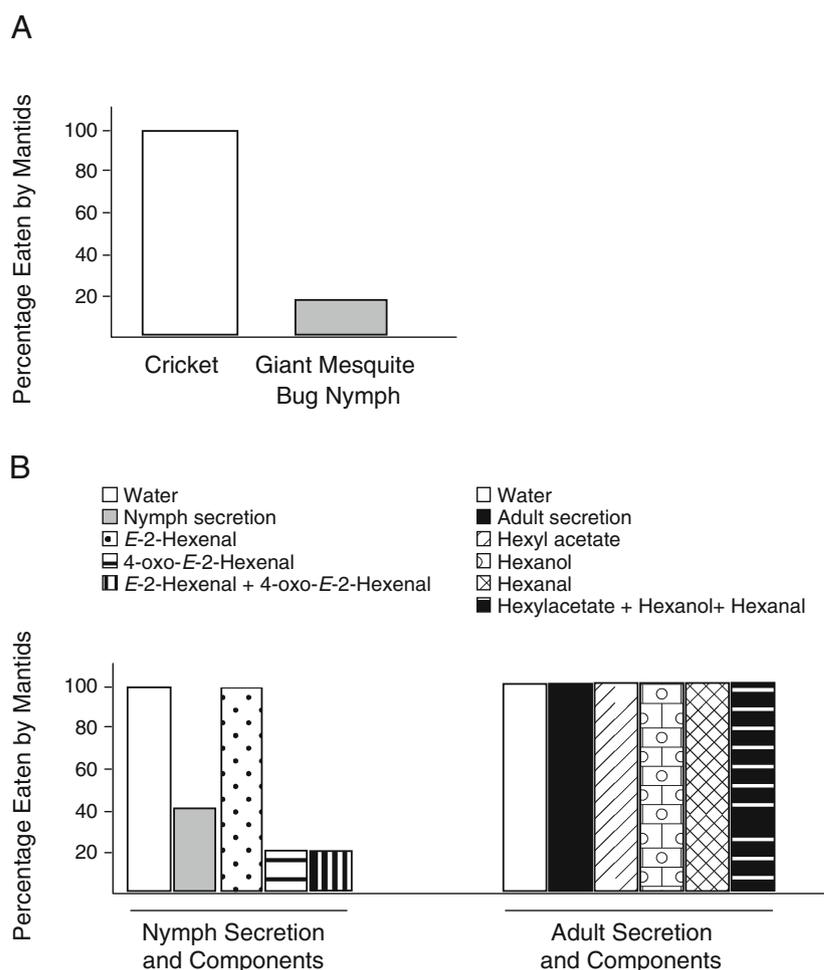


Fig. 4 Praying mantid responses to **a** nymph giant mesquite bug and **b** crickets treated with nymph secretion, adult secretion, and individual chemical components of the two secretions ($N=5$ mantids per treatment)



Crickets painted with adult bug secretion and all other compounds not listed above were always consumed by the mantids ($P>0.05$ for all comparisons; Fig. 4).

Discussion

Our chemical analyses revealed that giant mesquite bugs (*T. neocalifornicus*) nymphs and adults emit different compounds when disturbed. Nymphs emit (*E*)-2-hexenal and 4-oxo-(*E*)-2-hexenal, while adults emit hexyl acetate, hexanal, and hexanol. These results were confirmed with whole-body extracts of both nymphs and adults. All compounds are found in other heteropteran and even coreid species, and are produced in the metathoracic gland from fatty acids acquired from the host plant (Blum 1981; Aldrich 1988; Leal et al. 1994; Millar 2005). Chemical differences between life stages have been shown in other coreid species (Prestwich 1976; Aldrich 1988; Leal et al. 1994), but this is the first time it has been documented in the genus *Thasus*.

Our predator behavioral experiments demonstrated that nymphs were chemically protected from insect predators while adults were not (Fig. 4). California mantids failed to consume nymphs and crickets treated with nymph secretion, but did consume crickets treated with adult secretion. We attempted to test the mantids' responses to adult giant mesquite bugs, but mantids failed to orient or attack adults. Mantids did not seem to perceive adult mesquite bugs as potential prey, perhaps because of their size. However, further behavioral experiments are needed to test this interpretation. Of all compounds, 4-oxo-(*E*)-2-hexenal was particularly deterrent and toxic to mantids. Mantids often died after exposure to nymph secretion and to 4-oxo-(*E*)-2-hexenal even without consuming prey. Tarantulas (*Eurypelma* sp.) have also been shown to die after exposure to the vapors of the giant mesquite bug nymph secretion (De La Torre-Bueno and Ambrose 1936). 4-Oxo-(*E*)-2-hexenal is known for its mutagenic and cytotoxic qualities by reacting with deoxyguanosine (Kasai et al. 2005); however, this is, we believe, the first time it has been evaluated as a source of chemical defense against predators. Relative to 4-oxo-(*E*)-2-hexenal, the other

compounds did not deter mantid prey consumption. The lack of response by adult mesquite bugs to nymph alarm pheromones fits with the cryptic defensive strategy of adults. Differences in both chemical composition and behavior between life stages of giant mesquite bugs and the predator responses to these bugs and their compounds support the idea that the nymph compounds are particularly effective at deterring arthropod predators (Eisner 1970). Further research will explore the adaptive significance of this pattern and how concentration differences may affect predator response. The compounds may no longer be relevant defensive compounds in an adult giant mesquite bug because the predator species have changed.

Our mesquite bug behavioral experiments indicated that the secretions can be also used as an alarm signal to other conspecifics in the same life stage (Figs. 2 and 3). Nymphs dispersed from their aggregation when nymph secretions, (*E*)-2-hexenal or 4-oxo-(*E*)-2-hexenal, were placed in the arena. However, nymphs failed to disperse when they were exposed to adult secretion or any of the individual compounds found in the adult secretion. Adults followed a similar pattern of dispersing after experiencing adult secretion, hexyl acetate, or hexanal. Hexanol did not elicit a behavioral response by either nymphs or adults. Future research, however, should address the question whether behavioral responses change when concentrations of secretion compounds change. These compounds are known alarm pheromones in other heteropteran species (Aldrich 1988; Millar 2005). It is not unusual that the two life stages respond differently to the signals (Aldrich 1988), although there are examples of nymphs and adults responding to each other's alarm pheromones (e.g., *Leptoglossus zonatus*, Leal et al. 1994).

The described alarm pheromones also may be particularly important in the evolution of warning coloration and gregariousness in nymphs of the giant mesquite bug. Warning coloration is thought to facilitate evolution of gregariousness (Sillen-Tullberg 1988, 1993; Tullberg and Hunter 1995). Yet, there is still a high individual cost of being attacked after a group is discovered by a predator, especially when the group is small (Gamberale and Tullberg 1998). One way to mitigate this cost is for the individuals under attack to disperse after a predator encounter and reform another group at a different location. Future research should focus on the question if the alarm pheromone compounds found in the nymphs of giant mesquite bugs function in this way.

The giant mesquite bug is a predominant insect in the southeastern Arizona, yet little is currently known about its ecology and evolution. Here, we have described some aspects of its chemical ecology and behavioral responses. We have shown that a single compound, 4-oxo-(*E*)-2-hexenal, functions both as a deterrent to predators and an

alarm pheromone to conspecifics. Because of these multiple functions it may be a particularly interesting compound to study other members of the genus *Thasus*. We have also shown that the alarm pheromones in giant mesquite bugs are composed of multiple components and are different between nymphs and adults. The threat of predation is not communicated from one life stage to another. The adaptive reason explaining the need for multiple components in an alarm signal remains unexplored in this group.

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