2021

New Piperidine alkaloids from fire ants are not sequestered by the green and black poison frog (Dendrobates auratus)

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Chemoecology (2021) 31:391–396
https://doi.org/10.1007/s00049-021-00357-1

CHEMOECOLOGY

SHORT COMMUNICATION

Piperidine alkaloids from fire ants are not sequestered by the green and black poison frog (*Dendrobates auratus*)

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Received: 3 May 2021 / Accepted: 23 June 2021 / Published online: 3 July 2021

Abstract
Neotropical poison frogs possess alkaloid-based antipredator defenses which they sequester from a diet of arthropods such as oribatid mites and myrmicine ants. Alkaloid sequestration is still poorly understood and although several studies have examined its uptake, most experiments directly feed alkaloids to the frogs. Here, we examined the alkaloid uptake system in the poison frog species *Dendrobates auratus* by feeding it an alkaloid-containing prey item, the red imported fire ant *Solenopsis invicta* (Formicidae, Myrmicinae). Captive bred frogs were either fed live ants or fruit flies dusted with powdered ants for 4 months. Using GC–MS, we confirm that *S. invicta* contain previously described piperidine alkaloids known as solenopsins; however, none of these piperidine alkaloids was detected in the skin of *D. auratus*, suggesting the frogs are incapable of sequestering solenopsins from *S. invicta*. It is possible that *D. auratus* are unable to sequester fire ant piperidines due to their long hydrocarbon side chains, a feature that makes them structurally different than most known alkaloids in poison frogs.

Keywords Alkaloid sequestration · Chemical defense · Dendrobatidae · Feeding experiment · Myrmicinae · Solenopsin

Introduction

Chemical defenses are commonly used by organisms to deter predators, inhibit pathogens, and increase survivability (Berenbaum 1995; Hovey et al. 2018). In general, defenses are either synthesized de novo or sequestered, i.e., acquired from external sources and retained or stored in specialized structures (Duffey 1980; Mebs 2001; Savitzky et al. 2012). Sequestration from dietary sources has been described in a large number of animal lineages, including invertebrates (McPhail et al. 2001; Nishida 2002; Opitz and Müller 2009) and vertebrates (Dumbacher et al. 2009; Saporito et al. 2012; Savitzky et al. 2012). Among vertebrates, Neotropical poison frogs (Dendrobatoidea) are the most diverse and well-studied group and sequester alkaloid-based chemical defenses from their arthropod diet into specialized dermal glands (Saporito et al. 2010a, 2012). Over 500 alkaloids have been discovered in the skin of these frogs, yet many of the dietary sources for these alkaloids have not been identified (for reviews see Saporito et al. 2012, 2015; Santos et al. 2016) and the details on alkaloid uptake are not well understood (Santos et al. 2016; O'Connell et al. 2021). Dietary specialization appears to be a key evolutionary factor in sequestering defensive chemicals, and numerous studies have compared the alkaloids present in wild-caught frogs to those found in syntopic leaf-litter arthropods and/or frog stomach contents (Saporito et al. 2003, 2004, 2011; McGugan et al. 2016; Moskowitz et al. 2020). Most alkaloids sequestered by dendrobatids appear to be derived from oribatid mites and myrmicine ants (Saporito et al. 2007b, 2015; Jones et al. 2012), taxa that are known to comprise a large portion of dendrobatid frog diets (Donnelly 1991; Simon and Toft 1991; Caldwell 1996). Several studies have experimentally examined alkaloid uptake by feeding alkaloids to captive-bred poison frogs (Jeckel, Grant, and Saporito, unpub; Daly et al. 1994a, b; Sanchez et al. 2019; O'Connell et al. 2021); however, very few studies have fed alkaloid-containing arthropods directly to frogs (see Daly

Communicated by Günther Raspotnig.

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et al. 1992, 1994b). To date, only one published study has fed an alkaloid-containing ant to a dendrobatid poison frog. Daly et al. (1994b) fed the ant *Monomorium pharaonis*, which contains a pyrrolidine and indolizidine alkaloid, to the green and black poison frog *Dendrobates auratus* for 7 weeks. Interestingly, only the indolizidine was sequestered, whereas the pyrrolidine was not (Daly et al. 1994b), suggesting that there is selectivity in the alkaloid uptake system present in dendrobatid poison frogs.

In the present study, we aimed to further investigate the alkaloid uptake system in *D. auratus* by conducting a feeding experiment with the red imported fire ant *Solenopsis invicta* (Formicidae, Myrmicinae), a species known to contain a diversity of piperidine alkaloids. *Dendrobates auratus* is naturally found from Nicaragua to Colombia (Lötters et al. 2007), and *Solenopsis invicta* is also native to Central and South America, but has become invasive in many regions such as the southern USA and Caribbean (Morrison et al. 2004). Ants in the genus *Solenopsis* have been found in stomachs of dendrobatids (Born et al. 2010), and *S. invicta* contain piperidine alkaloids known as solenopsins (2-methyl-6-alkyl or alkenylpiperidines; MacConnell et al. 1970; Brand et al. 1972; Chen and Fadamiro 2009a), some of which are also found in poison frogs (Daly et al. 2005). To determine the extent to which solenopsins can be sequestered by *D. auratus*, we carried out a feeding experiment in which wild-caught *S. invicta* were fed to captive-raised *D. auratus*.

Materials and methods

Frog feeding experiment 1: live ants

Forty-two captive-bred adult *D. auratus* were kept as sets of 4 in 38 l tanks at 24–27 °C. Frogs were randomly assigned to treatment groups with males and females being assigned separately to maintain mixed pair housing. The control group contained 18 frogs and was fed with 10 pinhead house crickets (*Acheta domesticus*, fed with Fluker's© Cricket Food), while the experimental group containing 24 frogs was fed 10 live fire ants (*S. invicta,* 5 minor and 5 major class ants each). The ants were collected every 2–3 weeks from three separate colonies in Greenville, NC, USA, and fed with sugar water (1.5 parts filtered, distilled water and 1 part cane sugar) and Fluker's© Cricket Food. A single ant colony was used for all frogs per feeding, cycling through all colonies between feedings. All frogs were fed experimental meals in video monitored 15 × 18 × 12 cm clear plastic containers (with glass lids) twice a week for a total of 16 weeks. Frogs were allowed to acclimate for 30 min before introducing the prey. During the 25-min observation period, the number of prey consumed was recorded. To supplement the pinhead house crickets and ants, all frogs were also fed *Drosophila melanogaster* dusted with Repashy Calcium vitamin powder (Rapashy Ventures) four times a week and *Collembola* sp. ad libitum. Tanks were misted daily to maintain humidity.

Frog feeding experiment 2: ant powder

An additional 12 captive-bred adult *D. auratus* were used in a separate experiment in which frogs were fed fruit flies dusted with powder created from crushed fire ants. Fruit flies fed to the control group were dusted with vitamin powder (see above), whereas flies fed to the experimental group were dusted with ant powder composed of 1:1 powdered *S. invicta* and vitamin powder. Ants were ground into powder with mortar and pestle after freezing them with liquid nitrogen. Caste-composition was estimated 3250:250 minor and major workers per gram. The experimental group contained eight frogs, and the control group contained four *D. auratus*. Frogs were housed as described in experiment 1 (see above), and were fed fruit flies ad libitum four times a week during the 4-month experiment from August 2014 to December 2014.

Frog alkaloid extraction

Feeding experiments: live ants

Three frogs of the experimental group and two of the control group were randomly selected every other week (starting at week zero for the control group and at week two for the experimental group). A total of 42 frogs were killed during the 16-week experiments. Frogs were anesthetized with Benzocaine before their spinal cords were severed with stainless steel nips. Whole skins were dissected and stored individually in glass vials with Teflon coated lids containing 4 ml of 99.99% methanol at 4 °C until analysis. Methanol skin extracts were subject to an acid–base fractionation with a nicotine internal standard, following the methods of Saporito et al. (2010a) and Bolton et al. (2017). In brief, 1 ml of methanol was removed from each sample and combined with 50 µL of 1 N HCl and an internal standard of nicotine. Using a stream of nitrogen, this solution was concentrated to 100 µL and then diluted with 200 µL distilled H_2O. Extraction was performed four times, each with 300 µL of hexane. Next, the fraction was basified using saturated $NaHCO_3$. Extraction was continued using 300 µL of ethyl acetate, repeated a total of three times, and then dried with Na_2SO_4. Finally, solutions were evaporated to 100 µL using nitrogen. In addition, each sample was analyzed further. First, unaltered methanol extracts were directly analyzed, and second, frog skins were macerated using stainless steel shears, followed by direct analysis of methanol extracts.

Feeding experiments: ant powder

All frogs in the ant powder feeding experiments were euthanized after 4 months of feeding. Individual skins were collected and stored as described above, and each extract was analyzed initially by way of an acid–base fractionation, followed by analysis of the unaltered methanol extract, and then macerated methanol extract, as described above.

Ant alkaloid extraction

To characterize the major alkaloids, *Solenopsis invicta* from three separate colonies (see above) were collected in July 2013. From each location three samples were prepared, each consisting of five minor and five major caste ants. Ants were transferred to glass vials with Teflon coated lids containing 4 ml of 99.99% methanol. Methanol extracts were directly analyzed. To confirm the presence of alkaloids in the ant powder (see experiment 2), a small amount of powder was extracted using methanol and directly examined.

Alkaloid analysis

Alkaloid analysis was performed using gas chromatography–mass spectrometry (GC–MS), with a Varian Saturn 2100 T ion trap MS coupled to a Varian 3900 GC with a 30 m × 0.25 mm i.d. Varian Factor Four VF-5 ms fused silica column. Alkaloids were separated using a temperature program from 100 to 280 °C at a rate of 10 °C per minute with Helium as the carrier gas (1 ml/min). Each extract was analyzed with electron impact–mass spectrometry (EI–MS) as well as chemical ionization-mass spectrometry (CI–MS) with methanol as the reagent gas. A Varian MS Workstation v.6.9 SPI was used to generate MS spectra. Alkaloid identification was conducted using the NIST MS search 2.0 with the NIST 14 database, in combination with AMDIS, and by analysis of each mass spectra and comparison of diagnostic ion fragments with published data on frog alkaloids and *Solenopsis* fire ants (Daly et al. 2005; Chen and Fadamiro 2009a, b; Chen et al. 2010, 2012).

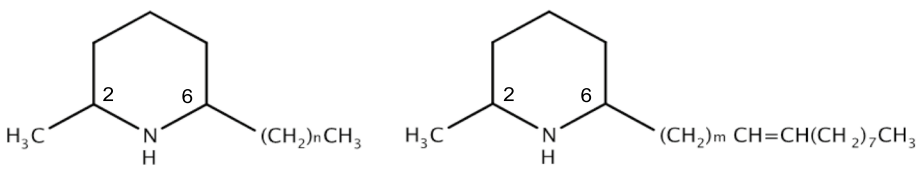
Results

Extracts of *S. invicta* individuals and *S. invicta* powder contained six major *cis*- and *trans*-2,6-disubstituted piperidine alkaloids, peaks **1–6** (Figs. 1, 2a), which were identified as *cis* and *trans*-C_{11}, *trans* $C_{13:1}$, *trans* C_{13}, *trans* $C_{15:1}$, *trans* C_{15}, and *trans* C_{17}, respectively. Four additional piperidines were also detected in minor quantities, but were not identified and are indicated by asterisks in Fig. 1a. Although ants and the fruit flies dusted with ant powder were readily eaten by the frogs, none of the piperidine alkaloids present in *S. invicta* was detected in any of the frog skins (Fig. 2b); however, benzocaine (euthanizing agent) was detected in all samples.

Discussion

Despite the fact that *S. invicta* and fruit flies dusted with *S. invicta* powder were readily consumed by the frogs in both experiments, *D. auratus* did not sequester any of the 2,6-disubstituted piperidine alkaloids into their skin. Given that frogs in the present feeding experiment were fed piperidines for several months, and in similar experiments alkaloids were sequestered within a few days or weeks (Daly et al. 1994b; O'Connell et al. 2021; Jeckel, Grant, and Saporito, unpub.), we conclude that the *D. auratus* used in our study were incapable of sequestering the piperidines produced by *S. invicta*. Over 20 2,6-disubstituted piperidines have been detected in dendrobatid poison frogs, yet they occur relatively rarely and usually in small quantities (Daly et al. 2005). Our findings suggest that this may be due to limitations in the alkaloid uptake and/or transport system, wherein 2,6-disubstituted piperidines are either not sequestered (as in the present study) or poorly sequestered by some dendrobatid species.

Defensive alkaloids vary greatly among and within poison frog species (Saporito et al. 2007a; Andriamaharavo et al. 2015; McGugan et al. 2016), including differences between



Peak
1 C_{11} n = 10
3 C_{13} n = 12
5 C_{15} n = 14
6 C_{17} n = 16

Peak
2 $C_{13:1}$ m = 10
4 $C_{15:1}$ m = 12

Fig. 1 Chemical structures of the six major 2,6-disubstituted piperidine alkaloids (solenopsins) detected in *Solenopsis invicta* (*cis*- and *trans*-stereoisomers are not indicated). Peak numbers 1–6 correspond to Fig. 2

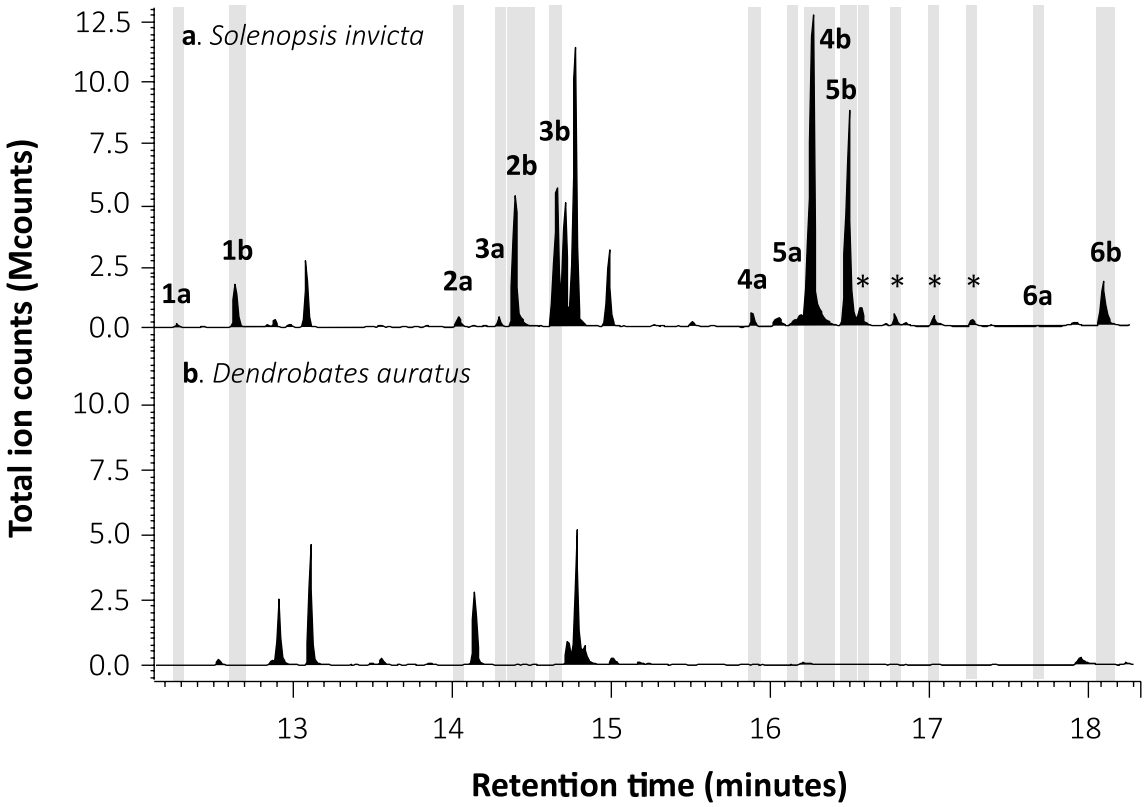


Fig. 2 Comparison of alkaloids in ants and frog skins. **a** GC trace of the six major *cis*- (1a–6a) and *trans*- (1b–6b) piperidine alkaloids present in *Solenopsis invicta* (C_{11}, $C_{13:1}$, C_{13}, $C_{15:1}$, C_{15}, C_{17}, respectively) and four additional piperidine alkaloids (*); and **b** the absence of these dietary provided piperidines in a *Dendrobates auratus* after 16 weeks of feeding (marked grey). Unlabeled peaks represent a combination of fatty acid methyl esters and phthalates

populations of *D. auratus* (Daly et al. 1992). Although much of this variation is likely explained by the availability of alkaloid-containing prey (Saporito et al. 2012; Prates et al. 2019; Moskowitz et al. 2020), differences in the ability or efficiency to sequester certain alkaloids appears to be important (Daly et al. 1994b; Mebs et al. 2014; Jeckel, Grant, and Saporito, unpub.). The ability to sequester alkaloids has a genetic basis and involves a wide spectrum of physiological adaptations, including means for alkaloid uptake, transport, and storage as well as mechanisms of auto-resistance (Santos et al. 2016; Sanchez et al. 2019; O'Connell et al. 2021; Jeckel, Grant, and Saporito, unpub.). If sequestration of certain alkaloids were to have a higher physiological cost (relative to other alkaloids), then selection may favor propensities against sequestering these alkaloids, resulting in reduced sequestration efficiency or a complete inability to sequester. The ability and efficiency of sequestration is likely dependent on the intrinsic chemical properties of the alkaloid, such as its chemical structure, stereochemistry (see Daly et al., 2003), and possibly the nature of its side chains. In the present study, the solenopsins detected in *S. invicta* possessed unbranched hydrocarbon side chains that were 11–15 carbons in length (*cis*- and *trans*-C_{11}, $C_{13:1}$, C_{13}, $C_{15:1}$, C_{15}, C_{17}), yet out of the hundreds of alkaloids discovered in poison frog skins, only four have been identified with side chains that are 11 carbons in length, all of which occur relatively rarely in frogs (Daly et al. 2005). Interestingly, all four of these alkaloids are piperidines, including the solenopsin C_{11} (2-methyl-6-undecylpiperidine, referred to as 2,6-disubstituted piperidine **253 J** in poison frogs) and three oxygenated piperidines that are structurally different than solenopsins (Daly et al. 2005). While small amounts of **253 J** have been detected in poison frogs, a feeding experiment with *cis*-2-methyl-6-undecylpiperidine (*cis*-**253 J**) did not result in sequestration by *D. auratus* (Daly et al. 1994b). Our results further support this finding, and suggest that **253 J** and other solenopsins are not sequestered by *D. auratus*; however, the presence of **253 J** in some poison frogs suggests that certain species are capable of sequestering this alkaloid, even if poorly, which will require further study.

Although the physiological mechanism of alkaloid sequestration in poison frogs is not well understood, they appear to be absorbed through the mucous membrane of the digestive tract and then transported through the lymphatic and/or circulatory system to specialized dermal granular glands for storage and secretion (Caty et al. 2019; Jeckel

et al. 2020; O'Connell et al. 2021). The absorption and transport of alkaloids is likely dependent on transporters, and both bile acid- and protein-based mechanisms have been proposed, including the protein transporter saxiphilin, which is known to bind the defensive chemical saxitoxin and possibly certain poison frog alkaloids (Clark et al. 2012; 2019; O'Connell et al. 2021). Therefore, the inability of *D. auratus* to sequester solenopsins may be due to the absence of a specific transporter (or transporters) necessary for the absorption and/or transport of these alkaloids, or solenopsins (with their long hydrocarbon side chains) may not be transported by available transporters. In addition, the ability of some species/populations to sequester certain alkaloids (such as **253 J**) suggests that specific transporters necessary for their absorption and/or transport may be differentially expressed, possibly related to the dietary alkaloids commonly available to them. Future experiments will help to determine the presence, function, and expression patterns of such transporters, as well as the factors responsible for their availability in different poison frog species and populations.

Acknowledgements We are thankful to Allison M. Anthony, Adam M. M. Stuckert and Miho Yoshioka for their help taking care of the frogs, and M. A. Nichols for his assistance in maintaining the GC–MS.

Funding Funding for this study was provided by an ECU THCAS Advancement Council Distinguished Professorship in the Natural Sciences and Mathematics to Kyle Summers, and a Kresge Challenge Grant awarded to JCU.

Availability of data and material Not applicable.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval The protocol used was approved by East Carolina University's Institutional Animal Care and Use Committee (AUP protocol #D288).

Consent to participate Not applicable.

Consent for publication All the authors declare their consent for publication.

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