J Chem Ecol
DOI 10.1007/s10886-015-0614-6

Differential Sequestration of a Cytotoxic Vismione from the Host Plant *Vismia baccifera* by *Periphoba arcaei* and *Pyrrhopyge thericles*

Ciara Raudsepp-Hearne[1,2,3] · **Annette Aiello**[1] · **Ahmed A. Hussein**[4,5] · **Maria V. Heller**[1,6] · **Timothy Johns**[2] · **Todd L. Capson**[1,2,7]

Received: 6 May 2014 / Revised: 29 June 2015 / Accepted: 30 July 2015
© Springer Science+Business Media New York 2015

Abstract We sought to compare the abilities of the specialist Lepidoptera *Pyrrhopyge thericles* (Hesperiidae) and the generalist *Periphoba arcaei* (Saturniidae) to assimilate three highly cytotoxic compounds from their larval host plant, *Vismia baccifera* (Clusiaceae) and to determine whether either insect discriminated in its assimilation of the compounds that are structurally similar but of variable cytotoxicity. Vismione B (**1**), deacetylvismione A (**2**), and deacetylvismione H (**3**) are cytotoxic compounds isolated from *V. baccifera*. Compound **1** was found in the 2nd and 3rd instars of *P. arcaei*, but not in the mature larvae or the pupae. *Pyrrhopyge thericles* assimilated trace quantities of compound **1** and deacetylvismione A (**2**), which were both found in the 3rd and 4th instars. In extracts of *V. baccifera*, compound **2** is present at levels approximately 6-fold greater than compound **1**, indicating that the generalist *P. arcaei* is capable of selectively sequestering cytotoxic compounds from its host plant. Compounds **1** and **2** show comparable cytotoxicities in three different cancer cell lines, suggesting that properties other than cytotoxicity are responsible for the selective sequestration of **1** by *P. arcaei.* This study represents the first time that sequestration of this class of compounds has been recorded in the Lepidoptera.

Keywords Cytotoxic · Sequestration · Aposematic · Clusiaceae · Lepidoptera · Saturniidae · Hesperiidae

✉ Todd L. Capson
capsont@gmail.com

1 Smithsonian Tropical Research Institute, P.O. Box 0843-03092, Balboa, Ancón, Republic of Panama

2 Department of Plant Science, McGill University, 21,111 Lakeshore Road, Ste. Anne de Bellevue, Quebec, Canada H9X 3V9

3 Present address: Department of Geography, McGill University, 845 Sherbrooke St. W., Montreal, Quebec, Canada H3A 2T5

4 Centro de Investigaciones Farmacognósticas de la Flora Panameña (CIFLORPAN), Facultad de Farmacia, Universidad de Panamá, Apartado 10767, Estafeta Universitaria, Panamá, Republica of de Panamá

5 Present address: University of the Western Cape, Private Bag X17, Bellville 7535, Republic of South Africa

6 Present address: National Bureau of Science, Technology and Innovation, City of Knowledge, Building 233, Panama, Republic of Panama

7 Present address: 1743 18th St., Washington, DC 20009, USA

Introduction

It has been proposed that the co-evolution or "arms race" between insect herbivores and plants has encouraged the development of defense mechanisms (*e.g.*, plant secondary metabolites) and fostered the diversification of both groups (Helson et al. 2009; Rausher 2001). Interactions between insects and their predators may also have led to the development of conspicuous aposematic coloration in insects that advertise their distastefulness or toxicity to visually oriented predators (Ruxton et al. 2007). We tested whether brightly colored, apparently aposematic Lepidoptera larvae were able to assimilate highly cytotoxic compounds from their host plant. We also asked whether there were differences between the sequestration of compounds by a generalist and a specialist aposematic herbivore when presented with a variety of cytotoxic compounds in a single host plant.

The tribe *Vismieae* (Clusiaceae) includes the genera *Vismia* Vand., *Harungana* Lam., and *Psorospermum* Spach. Plants of the genus *Vismia* produce a range of secondary metabolites, including triterpenoids, prenylated anthrones, anthraquinones, bianthraquinones, benzophenones, and

Published online: 28 August 2015

lignans (Hussein et al. 2003). Among the compounds found in species of *Vismia* are the vismiones, which are cytotoxic (Cassinelli et al. 1986; Hussein et al. 2003; Seo et al. 2000), antiprotozoal (Mbwambo et al. 2004), and antifeedant (Simmonds et al. 1985).

Vismia baccifera (L.) Triana & Planch is a common shrub in Panama, and is abundant in older clearings and along forest edges between sea level and 1000 m (D'Arcy 1987). The shrub is of variable height (2–22 m) with characteristic tomentose, orange branches and abundant yellow sap that is secreted from all vegetative structures especially during the rainy season. We previously reported the isolation of compounds vismione B (**1**), deacetylvismione A, (**2**), and deacetylvismione H (**3**) from *V. baccifera* (Fig. 1). We followed cytotoxicity against the NCI-H460 (lung), SF268 (central nervous system), and MCF-7 (breast) cell lines to isolate the bioactive compounds. The same study showed that compounds **1**–**3** are potent cytotoxins with IC_{50} values (the concentration of compound required to inhibit 50 % of cell growth) of approximately 4 μg/ml in MCF-7, NCI-H460, and SF268 cell lines for compounds **1** and **2**, and 0.5 μg/ml for compound **3** in the same cell lines (Hussein et al. 2003). Compound **1** had deterrent activity against noctuid lepidopteran larvae, whereas compound **2** was inactive in the same assay (Simmonds et al. 1985).

Through the monitoring of insect populations on *V. baccifera* at different localities, the larvae of *Pyrrhopyge thericles* Mabille, 1891 (Lepidoptera: Hesperiidae: Pyrrhopyginae) and *Periphoba arcaei* (Druce 1886) (Saturniidae: Hemileucinae) were shown to feed on the plant (AA and CRH unpublished observations). The larvae of both insect species are aposematic and presumably highly visible to potential avian predators, their bright appearance signaling the presence of defensive chemical compounds that are either biosynthesized or sequestered from a host plant (Blount et al. 2009; Bowers 1993; Nishida 2002; Opitz and Müller 2009).

Larvae of the skipper butterfly *P. thericles* (Fig. 2a), which occurs from Panama to Brazil, specialize on leaves of *Vismia* spp. In Panama, they have been found on *V. baccifera, V. billbergiana* Beurl., and *V. macrophylla* Kunth (AA unpublished). Pyrrhopygine caterpillars are banded with bright colors and are part of a vast group of aposematic, mimetic caterpillars (Burns and Janzen 2001). The larvae of *P. thericles* are bright red with yellow bands and long, white, erect setae.

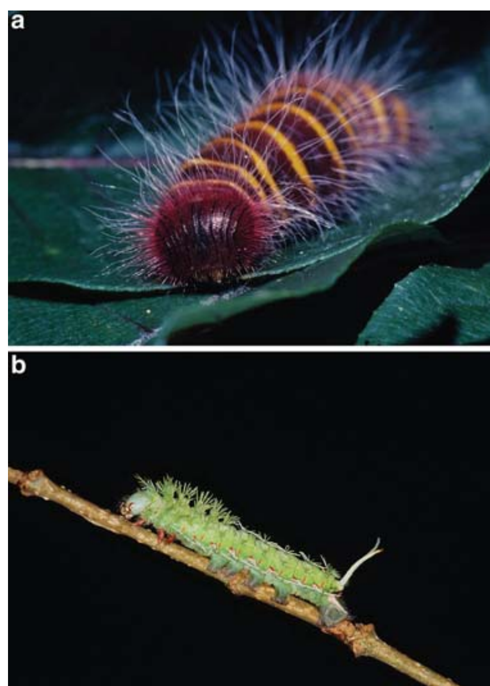
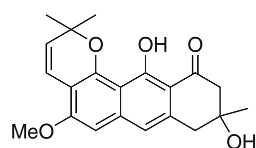


Fig. 2 **a** Final stage larva of *Pyrrhopyge thericles*. **b** Final stage larva of *Periphoba arcaei*

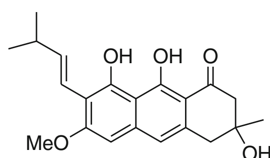
The pupa is bright red. The adult butterfly (Fig. 3 left) is black, with white wing margins, and has red-orange on the head and final segments of the abdomen. Ventrally, the wing bases are broadly white. They are rapid and powerful flyers and, given their lack of overall striking coloration, flight rather than chemistry may be their principal defense. The larvae eat exposed on leaves, but otherwise remain within a shelter formed by attaching two leaves together, one over the other. With modification, that same shelter serves as a pupation chamber (observations of AA).

Larvae of the giant silk moth, *P. arcaei* (Fig. 2b), which ranges from Mexico to Colombia (Janzen 1982), are generalists and in Panama have been reared on both young and old leaves of *V. baccifera, Quassia amara* L. (Simaroubaceae), *Anacardium excelsum* (Berero & Balb. *ex* Kunth) Skeels (Anacardiaceae) (AA and CRH unpublished observations), and in Costa Rica on at least eight different plant families (Janzen and Hallwachs 2003). The larvae are gregarious, and remain aggregated and sedentary upon the leaf surface for up to 60 days. First instars are bright red, but subsequent instars are increasingly aqua-green with lateral, slightly

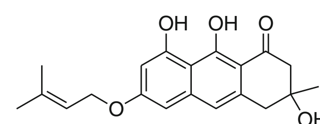
Fig. 1 Structures of vismiones isolated from *Vismia baccifera*



Vismione B (**1**)



Deacetylvismione A (**2**)



Deacetylvismione H (**3**)

Fig. 3 *Left*: Dorsal (*above*) and ventral (*below*) views of adult male *Pyrrhopyge thericles. Right*: Dorsal views of adult male (*above*) and female (*below*) of *Periphoba arcaei*

oblique, pink stripes and deep pink spiracles (Fig. 2b). There may be five or six larval stadia. All instars bear increasingly branched scoli with urticating setae that inject venom when their fragile tips break off in the skin of a potential predator (observations of AA). Though they are not as brilliantly patterned as the larvae of *P. thericles*, their aqua-green coloration, large size, large aggregations, and sedentary behavior render them conspicuous against their food plant, a suite of characteristics that strongly suggests unpalatability and the presence of biologically active secondary metabolites (Bowers 1993). At maturity, the larva ceases eating and develops a bright pink coloration prior to pupation. The larvae of many Lepidoptera undergo a pre-pupal color change, but given that many of them are hidden feeders, *e.g.*, numerous members of the Crambidae, we cannot speculate on the reason for the color change. The adult moth (Fig. 3 right) is variable in appearance but with a non-aposematic brown pattern. Its cryptic coloration combined with daytime immobility may be its main defense.

We studied the ability of *P. thericles* and *P. arcaei* to sequester the cytotoxic compounds from *V. baccifera*. While generalist herbivores are thought to have developed sensory skills to avoid dangerous plant chemicals, or physiological and biochemical mechanisms to overcome their toxic effects, sequestering specialists including the specialist Lepidoptera have gained an ability to incorporate the plant materials with relative impunity and without damaging target molecules (Nishida 2002). By extension, it would be expected that the specialist *P. thericles* would be better at assimilating toxic alkaloids from *V. baccifera* than the generalist *P. arcaei*.

Methods and Materials

Insect Material *Pyrrhopyge thericles* larvae were collected on *V. baccifera* at the Barro Colorado Nature Monument, Chagres National Park, and Altos de Campana National Park, all in the Republic of Panama. *Periphoba arcaei* was collected as an aggregation of eight larvae on *V. baccifera* in Altos de Campana National Park.

Insect Rearing All insects were raised in small wire cages with a paper towel disk and cut foliage of *V. baccifera* provided in excess, and were kept in Ziploc bags to maintain humidity levels (protocol of A. Aiello). Each day, larval fecula was removed and the paper towel was changed. For chemical analysis, larvae, pupae, and adults were killed by deep-freezing (−80 °C) and then stored in methanol at the same temperature. Some specimens were dissected to remove the gut completely, thus eliminating potential food plant residue. Adults were frozen after they had discharged the meconium. Samples of larval fecula and larval exuviae were collected and stored. To ensure that the presence of compounds **1** and **2** in insects was not an artifact of residual food plant in the insect gut, insects were starved for 36 h prior to analysis, yielding results that were qualitatively comparable to those obtained by removing the gut.

Food Plant Leaves of *V. baccifera* were collected in the vicinity of Panama City and in the national parks in which the insects were collected. A voucher specimen was deposited in the herbarium of the University of Panama (Aizprúa, Flores, & Araúz B3258). The identity of *V. baccifera* was confirmed by Professor Mireya Correa of the Smithsonian Tropical Research Institute and the University of Panama.

Extraction of Insects and Plants Insects and plants were homogenized with 5 ml of cold methanol for 5 min with a mortar and pestle followed by treatment with a Polytron homogenizer (Brinkman Instruments) at low temperature for 2 min or until the mixture appeared homogeneous. The mixture was filtered under vacuum through Whatman #4 filter paper, and the marc was washed with 50 ml of methanol. Finally, the mixture was filtered through Whatman #1 filter paper, and the extract was concentrated by rotary evaporation and stored at −80 °C.

Chemical Analysis and Cytotoxicity Measurements The identities of compounds **1** and **2** were confirmed by comparison with the authentic natural products by comparison of retention times by high performance liquid chromatography (HPLC), by UV spectroscopy, and by mass spectrometry. Chemical ionization mass spectrometry analyses were used to determine the molecular weights of compounds **1** and **2** and were carried out on a Kratos MS50TC instrument. HPLC analyses, which allowed us to separate the chemical components in insect and plant extracts, were performed on a Waters instrument (Milford, MA, USA) with a 600 E Quaternary pump with a photodiode array detector. HPLC analyses employed C18 Nova Pak columns (analytical: 4.6×250 mm,

4 micron; preparative: radial compression module kit, Nova-Pak, two 25 mm segments) utilizing acetonitrile (CH_3CN) and H_2O as solvents and with a linear gradient of 10–100 % CH_3CN over 0.5 h, monitoring at 254 nm. The retention times of compounds **1** and **2** were determined by injections of the individual compounds under the HPLC conditions used to analyze extracts from *P. thericles*, *P. arcaei*, and *V. baccifera.* The amounts of compounds **1** and **2** indicated in Fig. 3 were based on comparison with injections with known quantities of both compounds. The threshold for detection of compound **1** or **2** in our assay was approximately 0.1 μg per injection. UV spectra of compound **1** with showed peaks at 245, 307, and 408 nm comparable to literature values of 245, 305, and 410 nm (Delle Monache et al. 1979). UV spectra of compound **2** showed peaks at 239, 309, and 415 nm. The first publication to describe the chemical properties of compound **2** does not include UV data (Delle Monache et al. 1979).

Results

The ratio of compounds **1** and **2** in methanolic extracts of the leaves of *V. baccifera* was approximately 1:4 according to our HPLC analyses, in rough agreement with the ratio of 1:6 as determined by the isolation of the purified compounds from crude extracts of the plant (Hussein et al. 2003) (Fig. 4a). Deacetylvismione H (**3**) was not detected in measurable amounts in *V. baccifera* or in any stage of *P. arcaei* or *P. thericles* based on our HPLC analyses.

The 2nd and 3rd instars and pupa of *P. arcaei* yielded sufficient material for chemical analysis and yielded comparable results in our HPLC assay, showing the presence of compound **1**. Figure 4c shows the results obtained for the 3rd instar. Compound **2** was not detected in any of the life stages of *P. arcaei*. The identity of compound **1** in the 3rd instar was confirmed by mass spectrometry, which revealed a molecular ion (m/z=354) and a predominant ion resulting from loss of a methyl group (m/z=339), a loss which is characteristic for this class of compounds (Delle Monache et al. 1979). The fecula of *P. arcaei* showed compounds **1** and **2** in a ratio of approximately 1:2 (Fig. 4b).

The 3rd, 4th, and 5th instars, pupa, and adult of *P. thericles* provided sufficient material for analysis. Results from the 3rd and 4th instars were comparable, showing both compounds **1** and **2** at a ratio of approximately 1:1 but at levels near the threshold of detection in our HPLC assay (0.1 μg) (Fig. 4d). Analysis of the fecula of *P. thericles* revealed the presence of compounds **1** and **2** in a ratio of approximately 1:2 (Fig. 4e). No detectable quantities of either compound were detected in the 5th instar, pupae, or adults of *P. thericles* (data not shown).

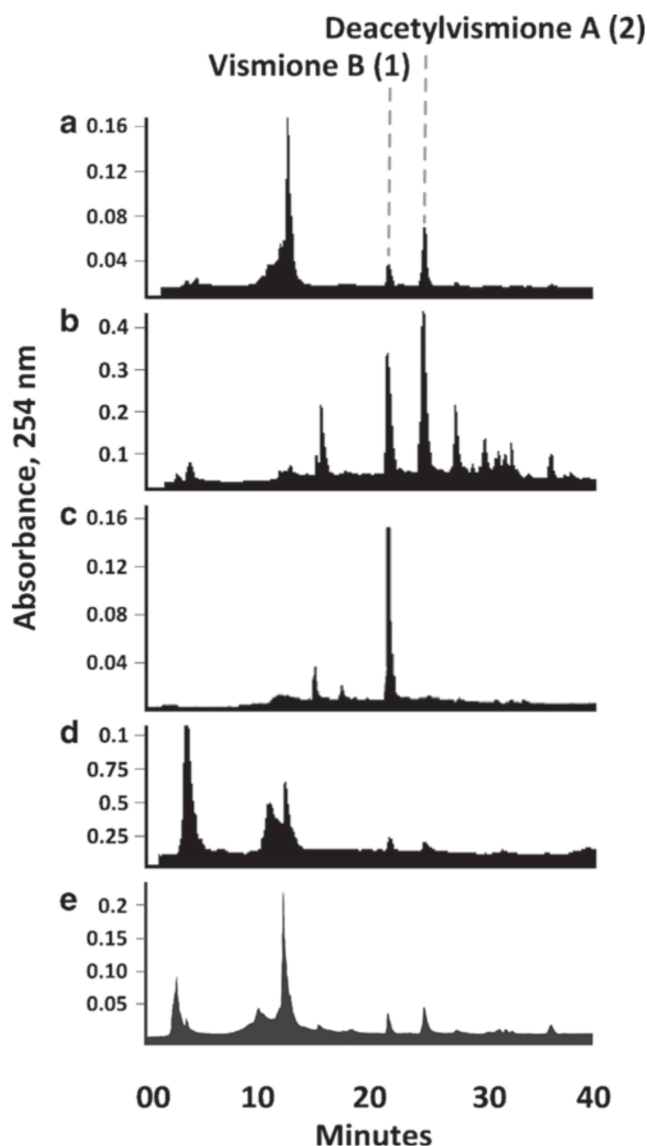


Fig. 4 HPLC traces of (**a**) crude extract of *Vismia baccifera*, (**b**) fecula of *Periphoba arcaei*, (**c**) 3rd instar of *P. arcaei*, (**d**) 3rd instar of *Pyrrhopyge thericles,* and (**e**) fecula of *P. thericles.* Quantities of vismione B (**1**) shown in panels **a**, **b**, **c**, **d**, and **e** are approximately 2.3, 3.7, 1.7, 0.1, and 0.4 μg, respectively. Quantities of deacetylvismione A (**2**) shown in panels **a**, **b**, **d**, and **e** are approximately 9.6, 7.4, 0.1, and 0.8 μg, respectively

Discussion

The observation that both aposematic generalist and specialist larvae feed on the same plant is not surprising given the results of a previous study that found no relationship between the evolution of aposematism in papilionid butterflies and the degree of diet specialization (Prudic et al. 2007). Analysis of the assimilation of compounds from the host plant, *V. baccifera,* by the generalist, *P. arcaei*, and the specialist, *P. thericles,* revealed two striking findings. First, cytotoxic compound **1** is sequestered far more effectively by larvae of *P. arcaei* than by larvae of *P. thericles.* Often specialists with

narrow host ranges are much more resistant to the effects of defensive compounds in their host plants than are generalists (Lampert and Bowers 2010; Nishida 2002; Reudler et al. 2011; Singer et al. 2004). Second, compound **1** was the only compound detected in *P. arcaei* (in the 2nd and 3rd instars). The similar cytotoxicities of **1** and **2**, and the absence of any detectable levels of compound **3,** the most cytotoxic of the three compounds, suggests that properties other than cytotoxicity are responsible for the assimilation of compound **1** by *P. arcaei.* Chemically, the only difference between **1** and **2** is the attachment of the isoprenoid side chain in **1** to C-8 *via* an oxygen bridge to form a pyran ring, which renders compound **1** more lipophilic than compound **2.** This structural feature may impact uptake since lipophilic compounds can cross membranes by simple diffusion (Nishida 2002). This property also may account for the observation that compound **1** has deterrent activity against noctuid lepidopteran larvae, while compound **2** does not (Simmonds et al. 1985).

The 1:2 ratio of compounds **1** and **2** in the fecula of both *P. arcaei* and *P. thericles,* compared to the ratio of 1:4 in *V. baccifera,* suggests the possible conversion of **2** to **1** by the insects. This conversion was predicted by Delle Monache et al. (1980) by plants of the genus *Vismia* based on the observation that **2** could be efficiently (84 % yield) converted to **1** by the dehydrogenating reagent, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone. A more compelling case for the insect-mediated conversion of compound **2** to **1** can be made for *P. arcaei* in light of the detection of compound **1** in the 2nd and 3rd instars of the insect. Our data do not exclude the possibility that compounds **1**, **2**, or **3** are otherwise assimilated by the insects used in this study, in whole or in part, and subsequently metabolized to compounds with significantly different retention times by HPLC and therefore not detected. Metabolism of sequestered compounds has been demonstrated in a number of Lepidoptera (Nishida 2002).

Compound **1** was not detected in the pupae of *P. arcaei,* which is consistent with other studies that have shown that toxic substances found in the aposematic life stages of insects are absent from life stages that do not have warning coloration (Boros et al. 1991; Nishida 2002). It has been proposed that changes in levels of toxic compounds at different life stages may be due to different ecological and evolutionary forces acting on the different life stages (Bowers 1993). Pupae are the most immobile and incapable of behavioral defense, though in most moth species they are within a protective cocoon, while larvae, though active, are much more sedentary than the winged adults. The potential autotoxic effects during metamorphosis, when massive reorganization of the internal organs, and high levels of cell growth and division occur, may account for the larvae ridding themselves of toxic compounds before pupating (Bowers 1993; Naberhaus et al. 2005; Nishida 2002). Nishida (2002) has noted that few classes of phytochemicals are sequestered by aposematic Lepidoptera, when compared to the broad range of potentially toxic plants utilized by this class of insects. Our results demonstrate the assimilation by Lepidoptera of a class of compounds not previously known to be sequestered by this group. We hypothesize that the compounds are used to deter predators, an idea that is indirectly supported by the combination of aposematic coloration and the highly cytotoxic nature of the compounds involved.

Acknowledgments We thank the Organization of American States, and the Office Québec- Amériques pour la Jeunesse for support for C.R.H. and the Fogarty International Center's International Cooperative Biodiversity Groups program (ICBG TW006634). We also thank two anonymous reviewers for their constructive comments on the original manuscript.

References

Blount JD, Speed MP, Ruxton GD, Stephens PA (2009) Warning displays may function as honest signals of toxicity. Proc R Soc B 276:871–877

Boros CA, Stermitz FR, Mcfarland N (1991) Processing of iridoid glycoside antirrinoside from *Maurandya antirrhiniflora* (Scrophulariaceae) by *Meris paradoxa* (Geometridae) and *Lepipolys species* (Noctuidae). J Chem Ecol 17:1123–1133

Bowers MD (1993) Aposematic caterpillars: life-styles of the unpalatable and warningly colored. In: Stamp N, Casey T (eds) Ecological and evolutionary constraints on caterpillars and the implications for pest management. Chapman & Hall, New York, pp 331–371

Burns JM, Janzen DH (2001) Biodiversity of pyrrhopygine skipper butterflies (Hesperiidae) in the Area de Conservación Guanacaste, Costa Rica. J Lepid Soc 55:15–43

Cassinelli G, Geroni C, Botta B, Delle Monache G, Delle Monache F (1986) Cytotoxic and antitumor activity of vismiones isolated from Vismieae. J Nat Prod 49:929–931

D'Arcy WG (1987) Flora of Panama, checklist and index, part I: the introduction checklist. Missouri Botanical Garden, Saint Louis

Delle Monache F, Ferrari F, Battista G, Bettolo M, Maxfield P, Cerrini S, Fedeli W, Gavuzzo E, Vaciago A (1979) Vismiones from *Vismia baccifera* Var. dealdata (H.B.K.): chemistry and x-ray structure determination. Gazz Chim Ital 109:301–310

Delle Monache F, Ferrari F, Marini Bettolo GB, Cuca Suarez LE (1980) Chemistry of the genus *Vismia*. Planta Med 40:340–346

Helson JE, Capson TL, Johns T, Aiello A, Windsor DM (2009) Ecological and evolutionary bioprospecting: using aposematic insects as guides to rainforest plants active against disease. Front Ecol Environ 7:130–134

Hussein A, Bozzi B, Correa M, Capson TL, Kursar TA, Coley PD, Solis PN, Gupta MP (2003) Bioactive constituents from three *Vismia* species. J Nat Prod 66:858–860

Janzen DH (1982) Guía para la identificación de mariposas nocturnas de la familia Saturniidae del Parque Nacional Santa Rosa, Guanacaste, Costa Rica. Brenesia 19:255–299

Janzen DH, Hallwach W (2003) Caterpillars, pupae, butterflies & moths of the ACG. http://janzen.sas.upenn.edu/index.html

Lampert EC, Bowers MD (2010) Host plant influences on iridoid glycoside sequestration of generalist and specialist caterpillars. J Chem Ecol 36:1101–1104

Mbwambo ZH, Apers S, Moshi MJ, Kapingu MC, Miert SV, Claeys M, Brun R, Cos P, Pieters L, Vlietinck A (2004) Anthranoid compounds

with antiprotozoal activity from *Vismia orientalis*. Planta Med 70: 706–710

Naberhaus I, Zintgraf V, Dobler S (2005) Pyrrolizidine alkaloids on three trophic levels - evidence for toxic and deterrent effects on phytophages and predators. Chemoecology 15:121–125

Nishida R (2002) Sequestration of defensive substances from plants by Lepidoptera. Annu Rev Entomol 47:57–92

Opitz SEW, Müller C (2009) Plant chemistry and insect sequestration. Chemoecology 19:117–154

Prudic LK, Oliver JC, Sperling FAH (2007) The signal environment is more important than diet or chemical specialization in the evolution of warning coloration. Proc Natl Acad Sci U S A 104: 19381–19386

Rausher MD (2001) Co-evolution and plant resistance to natural enemies. Nature 411:857–864

Reudler H, Biere A, Harvey JA, Nouhuys SV (2011) Differential performance of a specialist and two generalist herbivores and their parasitoids on *Plantago lanceolata*. J Chem Ecol 37:765–778

Ruxton GD, Speed MP, Broom M (2007) The importance of initial protection of conspicuous mutants for the coevolution of defense and aposematic signaling of the defense: a modeling study. Evolution 61:2165–2174

Seo EK, Wani MC, Wall ME, Navarro H, Mukherjee R, Farnsworth NR, Kinghorn AD (2000) New bioactive aromatic compounds from *Vismia guianensis*. Phytochemistry 55:35–42

Simmonds MSJ, Blaney WM, Delle Monache F, Marquina Mac-Quhae M, Marini-Bettolo GB (1985) Insect antifeedant properties of anthranoids from the genus *Vismia*. J Chem Ecol 11:1595–1599

Singer MS, Carriere Y, Theuring C, Hartmann T (2004) Disentangling food quality from resistance against parasitoids: diet choice by a generalist caterpillar. Am Nat 164:423–429