SHORT COMMUNICATION

Orange/lemon-scented beetles: opposite enantiomers of limonene as major constituents in the defensive secretion of related carabids

Athula B. Attygalle · Xiaogang Wu ·
David R. Maddison · Kipling W. Will

Abstract The major constituent in the pygidial gland defensive fluid of the carabid beetle *Ardistomis schaumii* is (R)-(+)-limonene, whereas that of *Semiardistomis puncticollis* is (S)-(−)-limonene. This was an unanticipated result, since it is not very common to find the opposite enantiomers of the same compound among the secondary metabolites of related species. Moreover, the glandular liquid of *A. schaumii* contains 1,8-cineole, and that of *S. puncticollis* has β-pinene, β-phellandrene, sabinene, and *p*-cymene. Of about 500 carabid species that have been chemically investigated, this is the first report of the presence of such complex mixtures of monoterpenes in their defensive secretions.

Keywords Carabid beetle · β-pinene · *p*-cymene · Terpenes · *Semiardistomis puncticollis* · *Ardistomis schaumii* · Limonene

A. B. Attygalle (✉) · X. Wu
Department of Chemistry,
Chemical Biology and Biomedical Engineering,
Stevens Institute of Technology,
Hoboken, NJ 07030, USA
e-mail: athula.attygalle@stevens.edu

D. R. Maddison
Department of Entomology, University of Arizona,
Tucson, AZ 85721, USA

K. W. Will
Department of Environmental Science, Policy and Management,
Division of Organisms and Environment, University of California,
Berkeley, CA 94720, USA

Introduction

One of the most species-rich families of beetles, composed of around 33,000 described species (Lorenz 2005), is the Carabidae. They are well known to produce a plethora of defensive compounds which may have contributed to their diversification (Erwin 1985). Information on the defensive chemistry of over 500 carabid species show that their defensive secretions consists mainly of hydrocarbons, acids, quinones, aliphatic ketones, and aldehydes, and a variety of other organic compounds (Will et al. 2000; Blum 1981; Weatherston and Percy 1978). Many higher plants produce essential oils consisting mainly of terpenes to protect themselves from herbivores. Terpenes are well known from some insects such as ants (Attygalle and Morgan 1984; Blum 1981), hemipteran bugs, (Aldrich et al. 1979), sawflies (Eisner et al. 1974), and termites (Quintana et al. 2003; Moore 1964). However, only a single terpene has been reported from carabid beetles (Moore and Brown 1979), although they are known to produce over 230 compounds (Attygalle and Will unpublished, Francke and Dettner 2005).

Clivinini is a world-wide tribe of carabid beetles abundant in tropical and temperate zones, with approximately 60 genera and nearly 1,200 described species (Lorenz 2005); however, species from only three genera of this tribe have been investigated for their defensive chemistry. Three species of *Clivina* Latrelle have been found to produce benzoquinones (Schildknecht et al. 1968a; Schildknecht et al. 1968b, Moore and Wallbank 1968, Kanehisa and Murase 1977), whereas *Schizogenius lineolatus* (Say) produces unsaturated carboxylic acids (Will et al. 2000) and *Setodyschirius wilsoni* (Sloane) produces exclusively aliphatic ketones and aldehydes (Moore and Brown 1979). Members of Clivinini are

recognized by their pedunculate body-form and modified fore-legs, apparently adapted to their fossorial habits. This general body-form and other characteristics are also found in two other carabid tribes, Scaritini and Rhysodini. Scaritini are generally considered to be phylogenetically related to Clivinini, and Rhysodini are considered to be related to Clivinini by some others (e.g., Bell 1994). However, the exact relationships of these taxa vary widely according to different authors and taxonomic character systems (Maddison et al. 1999; Fedorenko 1997, Bell 1994; Jeannel 1946).

In contrast to the variety of defensive compounds known from clivinines, all Scaritini (in the restricted sense) produce saturated and unsaturated carboxylic acids such as methacrylic and tiglic acids (Moore and Wallbank 1968; McCullough 1969, McCullough 1972; Kanehisa and Murase 1977, Moore 1979; Kanehisa and Kawazu 1982; Davidson et al. 1989). There are no published records of the pygidial gland secretions for Rhysodini; however, two North American species we have sampled exclusively produce the same classes of compounds as those found in Scaritini (Attygalle and Will unpublished).

Two major groups are usually recognized within clivinines: Dyschiriina, which are a group supported by a variety of synapomorphies (e.g., having a single secretory cell per reservoir) and the remaining clivinines, which are a heterogeneous assemblage, and probably not a natural group. Some of these clivinines are potentially closely allied to Dyschiriina or even Scaritini. Because the entire complex of Scaritini, Rhysodini and Clivinini are heterogeneous for major pygidial gland compounds, these data may offer insight into the relationships among the members. The taxa reported herein are from two genera, *Ardistomis* Putzeys and *Semiardistomis* Kult. These genera share similar morphological characteristics [e.g., form of the elytral-abdomen apical locking mechanism, fully divided meta-coxae and form of the elytral humeral border (Nichols 1988)] with other taxa that may form a monophyletic group within clivinines.

Material and methods

Insect collection and sample preparation

Individual beetles of *Ardistomis schaumii* Leconte were collected in South Carolina and Florida; specimens of an unidentified *Ardistomis* species were collected from Punta Cana, La Altagracia Province, Dominican Republic, and specimens of *Semiardistomis puncticollis* (Dejean; Supplementary Figure 1) were collected in Florida and Nebraska.

Voucher specimens are deposited in the Essig Museum of Entomology, University of California, Berkeley, CA, and the Oregon State Arthropod Collection, Oregon State University, Corvallis, OR. Beetles were reared on damp sand, or sphagnum, and fed with commercial dog food. Whole gland reservoirs were excised and immediately placed in reaction vials with solvent for analysis or placed in a capillary tube and sealed. Sealed tubes were then shipped to Stevens Institute of Technology, Hoboken, for analysis.

Chemical analyses

Authentic samples of 2-methyl-1,4-benzoquinone, (R)-(+)-limonene, (S)-(−)-limonene, 1,8-cineole, β-pinene, β-phellandrene, sabinene, and p-cymene were available in our laboratory collection of chemicals. Excised pygidial glands were crushed and extracted with hexane or dichloromethane (20 μL). One-microliter aliquots of the extract was subjected to gas chromatography-mass spectrometry (GC-MS) analysis on a Shimadzu GCMS-QP5050A instrument fitted with either an XTI-5-coated (0.25 μm) fused silica capillary column (30 m×0.25 mm) [The oven temperature was held at 30°C for 4 min, increased at 4°C/min to 200°C, and then increased to 260°C at a rate of 20°C/min],
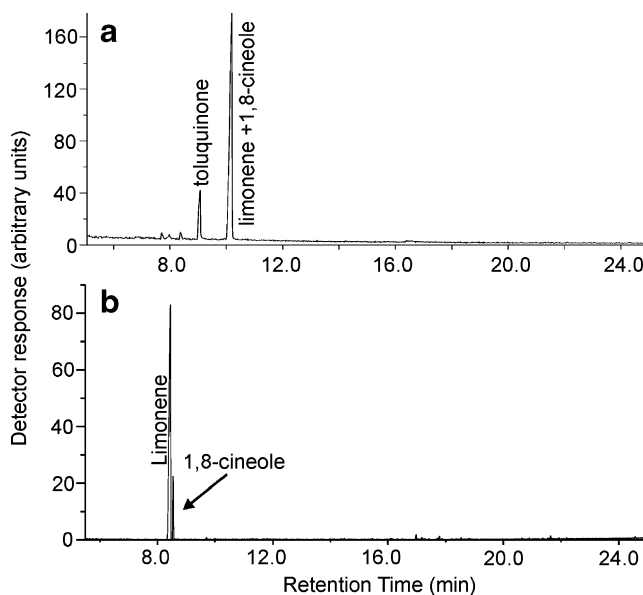

Fig. 1 Reconstructed gas chromatograms obtained from glandular volatiles of *Ardistomis schaumii*. A dichloromethane extract was analyzed by GC-MS using (**a**) an HP 5989A mass spectrometer linked to an HP 5890 gas chromatograph fitted with an HP-1-coated fused silica capillary column (25 m×0.2 mm, 0.11 μm; the oven temperature was held at 30°C for 3 min and increased at 8°C/min to 270°C), or (**b**) a Shimadzu GCMS-QP5050A gas chromatograph-mass spectrometer fitted with a ZB-FFAP-coated (0.25 μm) fused silica capillary column (30 m×0.25 mm; the oven temperature was held at 30°C for 2 min and increased at 8°C/min to 240°C)

or a Zebron Free Fatty Acid Phase-coated (0.25 μm) fused silica capillary column (30 m×0.25 mm) [The oven temperature was held at 30°C for 2 min and increased at 8°C/min to 240°C]. Some analyses were carried out using an HP 5989A mass spectrometer linked to an HP 5890 gas chromatograph fitted with an HP-1 (0.11 μm) fused silica capillary column (25 m× 0.2 mm) [The oven temperature was held at 30°C for 3 min and increased at 8°C/min to 270°C].

For gas chromatography with flame ionization detection (GC-FID) analysis of limonene enantiomers an Agilent 6890 series GC system fitted with a Cyclodex-B-coated (0.25 μm) fused silica capillary column (30 m×0.25 mm; J&W Scientific) was used. The oven temperature was held at 40°C for 2 min and increased at 4°C/min to 230°C. Further analyses were carried out by installing the Cyclodex-B column in the Shimadzu GCMS-QP5050A system. The oven temper-

Table 1 Relative amounts of compounds found in the defensive secretion of three clivinine beetles ($N=3$ for each species)
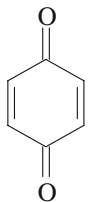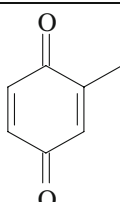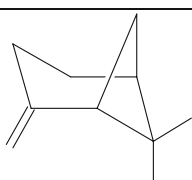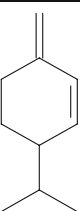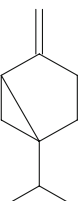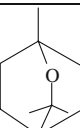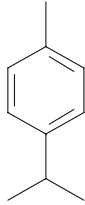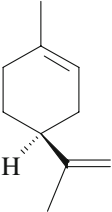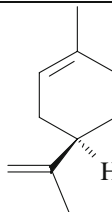
Compound	Structure	Relative Abundance		
		Ardistomis schaumii	*Semiardistomis puncticollis*	*Ardistomis sp.*
1,4-benzoquinone		-	7.4	3.1
2-methyl-1,4-benzoquinone		19.4	19.5	46.9
β-pinene		-	4.5	-
β-phellandrene		-	1.9	-
sabinene		-	3.1	-
1,8-cineole		10.7	-	-

Table 1 (continued)

p-cymene		-	0.8	3.8
(*S*)-(-)-limonene		3	100	trace
(*R*)-(+)-limonene		100	25	100

ature was held at 80°C for 2 min and increased at 4°C/min to 120°C, and then increased to 230°C at a rate of 20°C/min.

Results and discussion

Gas chromatographic mass spectrometric analysis of a solvent extract prepared from the pygidial glands of *Ardistomis schaumii* on a nonpolar GC phase showed two major peaks (Fig. 1a). The first-eluting peak represented 2-methyl-1,4-benzoquinone (toluquinone), a well-known constituent from many carabid and other arthropod defensive secretions (Blum 1981; Will et al. 2000). The mass spectrum corresponding to the major peak however appeared similar to that of a monoterpene. To the best of our knowledge, terpenes have been reported from carabid secretions only on one occasion by Moore and Brown (1979), who reported iridodial in the defensive secretion of *Setodyschirius wilsoni*. To obtain a better chromatographic separation of the defensive mixture, the extract from *A. schaumii* was reanalyzed on a polar column (Fig. 1b). The results confirmed that the major chromatographic peak in Fig. 1a represents a composite mixture of two compounds. The minor constituent was identified as 1,8-cineole (1) by its mass spectrum and a comparison of its gas chromatographic retention with that of an authentic sample. Similarly, the major peak was identified as limonene. Although the presence of monoterpenes in beetle secretions is common (Francke and Dettner, 2005), the discovery of limonene and 1,8-cineole as major constituents was unexpected because both compounds have not been reported before from carabid beetles. Previously, 1,8-cineole has been identified from the pygidial gland of staphylinid beetles such as *Stenus coma* Leconte (Schildknecht 1970; Schildknecht et al. 1976). 1,8-Cineole has been implicated to function as a propellant in water for these beetles (Schildknecht 1970). In fact, several other terpenes have been identified from staphylinid defensive substances (Wheeler et al. 1972; Bellas et al., 1974).
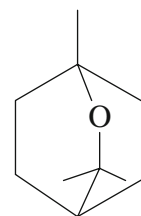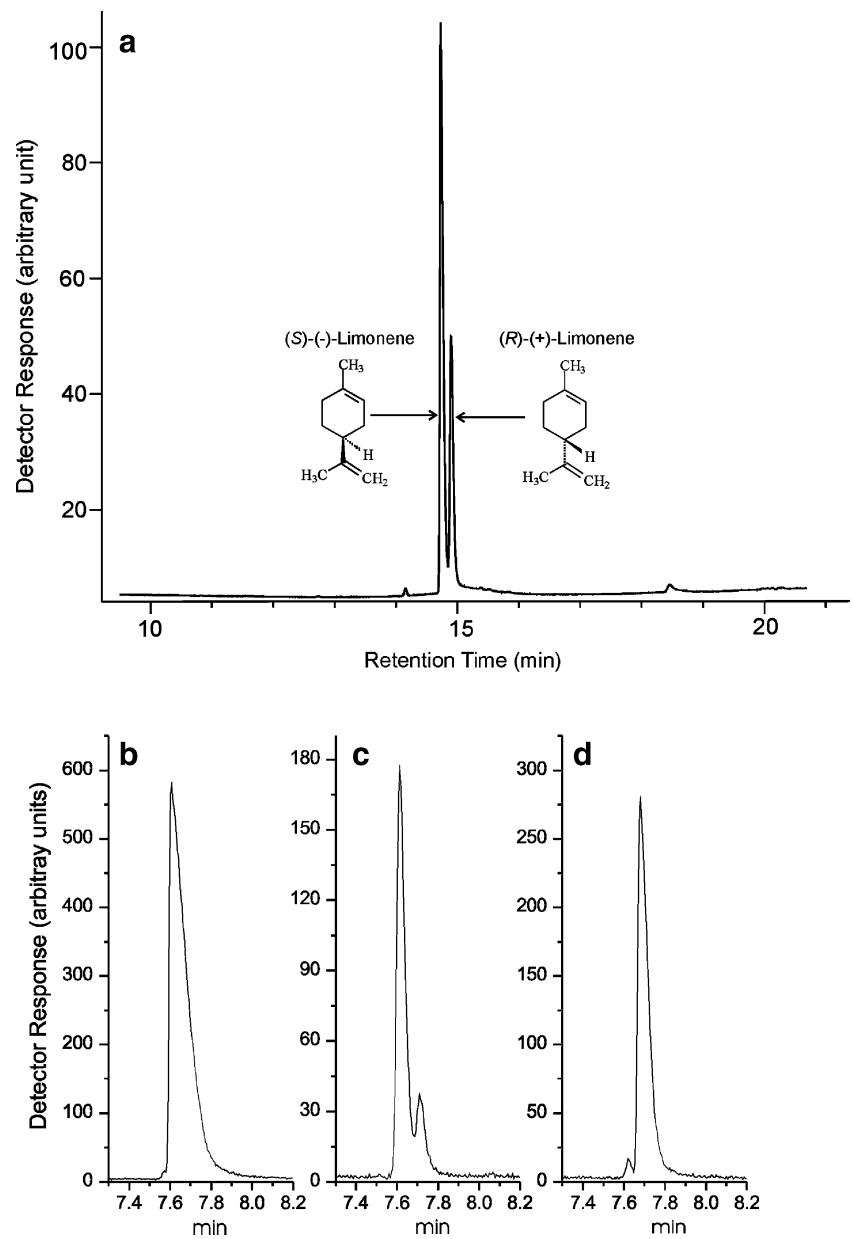

$$(1)$$

The presence of limonene and other monoterpenes is not restricted to *A. schaumii*. Analysis of pygidial glands contents of two other clivinines, *S. puncticollis* and *Ardistomis* sp. by GC-MS, showed the presence of several monoterpenes (Table 1) none of which have been reported from carabids previously.

Limonene, a well-known constituent in essential oils of several plants, is known to function as an insect repellent

(Ibrahim et al. 2001) as well as a repellent of some birds [e.g., Starlings (Clark 1997)] and mammals such as deer (Vourc'h et al. 2002)]. This monoterpene hydrocarbon is present in nature as two enantiomers (2, 3). Since the two enantiomers of limonene have different fragrances, they are used as specific blends in different consumer products (Braddock et al. 1995). In general, (R)-(+)-limonene is the dominant enantiomer in most plants that produce limonene, although the resin from a few plants such as *Pinus strobus* L. contains significant amounts of the (−)-enantiomer (Wang et al. 1997).
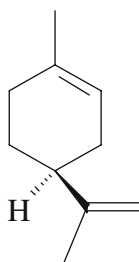
(2)

(S)-(-)-limonene
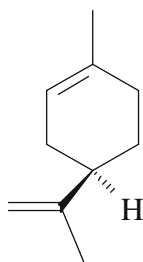
(3)

(R)-(+)-limonene

We examined a synthetic mixture made of 70% (S)-(−)-enantiomer and 30%(R)-(+)-enatiomer on a gas chromatographic column coated with Cyclodex B. Figure 2a shows that the two enantiomers of limonene can be separated by chiral gas chromatography, and the first eluting isomer is (S)-(−)-limonene. When a glandular extract of *A. schaumii* was analyzed by chiral gas chromatography, it was revealed that the limonene in the gland consisted essentially of the (R)-(+)-isomer (Fig. 2d, Table 1). However, a small amount of the opposite enantiomer is also present since a very small peak that corresponded to the retention time of (S)-(-)-isomer was also observed. Similar results were obtained from a sample obtained from *Ardistomis* sp. It is generally accepted that enantiomeric composition of chiral compounds of insects are species specific (Mori 1998a, b, 2007). Interestingly, combined GC-FID and GC-MS analyses on the Cyclodex-B-coated column showed that the major limonene enantiomer of the glandular extracts of *S. puncticollis*, is the (S)-(−)-enantiomer (Table 1), and that of *A. schaumii,* is the (R)-(+) enantiomer.

Terpenoid compounds have been reported mainly from defensive secretions of phytophagous insects (Weatherston and Percy, 1978), although it is not clear whether they sequester the compounds or produce them de novo. Adults and larvae of clivinine beetle investigated here are not phytophagous; therefore, it is unlikely that these terpenes are sequestered from the diet of the clivinines.

Many arthropod use benzoquinones as repellents. It is also known that benzoquinones are generally accompanied by hydrocarbons which have been speculated to serve as solvents for uptake of the quinones which are crystalline at room temperature (Wu et al. 2007; Eisner et al. 2000). Apparently nonpolar compounds facilitate the transport of active polar compounds through the waxed epicuticle of enemy arthropods. It is very likely that limonene acts as a permeation enhancer for benzoquinone. For example, it has been demonstrated that the transdermal delivery of acetylsalicylic acid, a potent antithrombotic drug, via skin patches can be significantly improved by admixing it with limonene (McAdam et al. 1996).

Our chemical results are in part phylogenetically informative. A close relationship of ardistomine genera is supported by the common occurrence of limonene. However, as the ardistomine taxa have both terpenes (characteristic for dyschiriines) and quinones (found in Clivina), their phylogenetic relationships within Clivinini cannot be determined using defensive chemical data alone. Because the presence of quinones and/or terpenes is common in Clivinini, it is likely to be a shared derived characteristic (synapomorphic) for the tribe, perhaps exclusive of Schizogenius. Unsaturated carboxylic acids as primary compounds is a feature found in Scaritini, Rhysodini, and Schizogenius Putzeys (Clivinini). The presence of these acids does not appear to be a synapomorphy for these taxa (Will et al. 2000; Attygalle et al. 2004). Consequently, defensive chemical data are not sufficiently informative to ascertain the relationships of Scaritini and Rhysodini relative to Clivinini.

References

Aldrich JR, Blum MS, Lord HA, Evans PH, Burkhard DR (1979) Novel exocrine secretions from two species of scentless plant bugs (Hemiptera: Rhopalidae). Entomol Exp Appl 26:323–331

Attygalle AB, Morgan ED (1984) Chemicals from the glands of ants. Chem Soc Rev 13:245–278

Attygalle AB, Wu X, Ruzicka J, Rao S, Garcia S, Herath K, Meinwald J, Maddison DR, Will KW (2004) Defensive chemicals of two species of *Trachypachus* Motschulski. J Chem Ecol 30:577–588

Bell RT (1994) Beetles that cannot bite: Functional morphology of the head of adult Rhysodines (Coleoptera: Carabidae or Rhysodidae). Can Entomol 126:667–672

Bellas TE, Brown WV, Moore BP (1974) The alkaloid actinidine and plausible precursors in defensive secretions of rove beetles. J Insect Physiol 20:277–280

Blum MS (1981) Chemical defenses of arthropods. Academic Press, New York

Braddock RJ, Cadwallader KR (1995) Fruit flavors, biogenesis characterization and authentication. ACS Symp Ser 596:142–148

Clark L (1997) Dermal contact repellents for Starlings: foot exposure to natural plant products. J Wildl Manage 61:1352–1358

Davidson B, Eisner T, Witz B, Meinwald J (1989) Defensive secretion of the carabid beetle *Pasimachus subsulcatus*. J Chem Ecol 15:1689–1697

Eisner T, Johnessee JA, Carrel J (1974) Defensive use by an insect of a plant resin. Science 184:996–999

Eisner T, Aneshansley DJ, Eisner M, Attygalle AB, Alsop D, Meinwald J (2000) Spray mechanism of the most primitive bombardier beetle (*Metrius contractus*). J Experim Biol 203:1265–1275

Erwin TL (1985) The taxon pulse: a general pattern of lineage radiation and extinction among carabid beetles. In: Ball GE (ed) Taxonomy, phylogeny, and zoogeography of beetles and ants: a volume dedicated to the memory of Philip Jackson Darlington Jr. 1904–1983. W. Junk, The Hague, pp 437–472

Fedorenko DN (1997) Reclassification of world Dyschiriini, with a revision of the palaearctic fauna (Coleoptera: Carabidae). Coleopt Bull 51:255–256

Francke W, Dettner K (2005) Chemical signalling in beetles. Top Curr Chem 240:85–166

Ibrahim MA, Kainulainen P, Aflatuni A, Tiilikkala K, Holopainen JK (2001) Insecticidal, repellent, antimicrobial activity and phytotoxicity of essential oils: With special reference to limonene and its suitability for control of insect pests. Agric Food Sci Finl 10:243–259

Jeannel R (1946) Coléoptères carabiques de la région Malgache (premiere partie). Faune de l'Empire Français 6:1–372

Kanehisa K, Kawazu K (1982) Fatty acid components of the defensive substances in acid-secreting carabid beetles. Appl Ent Zool 17:460–466

Kanehisa K, Murase M (1977) Comparative study of the pygidial defensive systems of carabid beetles. Appl Ent Zool 12:225–235

Lorenz W (2005) A systematic list of extant ground beetles of the world (Coleoptera "Geadephaga": Trachypachidae and Carabidae, incl. Paussinae, Cicindelinae, Rhysodinae). Published by author, Tutzing

Maddison DR, Baker MD, Ober KA (1999) Phylogeny of carabid beetles as inferred from 18S ribosomal DNA (Coleoptera, Carabidae). Syst Entomol 24:103–138

McAdam B, Keimowitz RM, Maher M, Fitzgerald DJ (1996) Transdermal modification of platelet function: an aspirin patch system results in marked suppression of platelet cyclooxygenase. J Pharmacol Exp Ther 277:559–564

McCullough T (1969) Chemical analysis of the scent fluid of *Pasimachus californicus* and *P. duplicatus* (Coleoptera: Carabidae). Ann Entomol Soc Am 62:1492

McCullough T (1972) Analysis of the defensive scent fluid of *Pasimachus elongatus* (Coleoptera: Carabidae). Ann Entomol Soc Am 65:772

Moore BP (1964) Volatile terpenes from Nasutitermes soldiers (Isoptera: Termitidae). J Insect Physiol 10:371–375

Moore BP (1979) Chemical defense in carabids and its bearing on phylogeny. In: Erwin TL, Ball GE, Whitehead DR, Halpern AL (eds) Carabid beetles: their evolution, natural history, and classification. W. Junk, The Hague, pp 193–203

Moore BP, Brown WV (1979) Chemical composition of the defensive secretion in *Dyschirius* Bonelli (Coleoptera: Carabidae: Scaritinae) and its taxonomic significance. J Austr Entomol Soc 18:123–125

Moore BP, Wallbank BE (1968) Chemical composition of the defensive secretions in carabid beetles and its importance as a taxonomic character. Proc Roy Entomol Soc London (B) 37:62–72

Mori K (1998a) Semiochemicals—Synthesis, stereochemistry, and bioactivity. Eur J Org Chem 1479–1489

Mori K (1998b) Chirality and insect pheromones. Chirality 10:578–586

Mori K (2007) Significance of chirality in pheromone science. Bioorg Med Chem 15:7505–7523

Nichols SW (1988) Systematics and biogeography of West Indian Scaritinae (Coleoptera: Carabidae), unpublished PhD. Thesis. Department of Entomology. Cornell University, Ithaca, NY.

Quintana A, Reinhard J, Faure R, Uva P, Bagnères A-G, Massiot G, Clèment J-L (2003) Interspecific variation in terpenoid composition of defensive secretions of European Reticulitermes termites. J Chem Ecol 29:639–652

Schildknecht H (1970) The defensive chemistry of land and water beetles. Angew Chem Int Ed Engl 9:1–9

Schildknecht H, Maschwitz U, Winkler H (1968a) Zur Evolution der Carabiden-Wehrdruesensekrte ueber Athropoden-Abwehrstoffe XXXII. Naturwissenschaften 55:112–117

Schildknecht H, Winkler H, Maschwitz U (1968b) Ueber Arthropoden-Abwehrstoffe: XXXI. Vergleichend chemische Untersuchungen der Inhaltsstoffe der Pygidialwehrblasen von Carabiden. Z Naturforsch B 23:637–644

Schildknecht H, Berger D, Krauss D, Connert J, Gehlhaus J, Essenbreis H (1976) Defense chemistry of *Stenus comma* (Coleoptera: Staphylinidae). LXI J Chem Ecol 2:1–11

Vourc'h G, de Garine-Wichatitsky M, Labbé A, Rosolowski D, Martin J-L, Fritz H (2002) Monoterpene effect on feeding choice by deer. J Chem Ecol 28:2411–2427

Wang X, Liu Y-S, Nair UB, Armstrong DW, Ellis B, Williams KM (1997) Enantiomeric composition of monoterpenes in conifer resins. Tetrahedron Asymmetr 8:3977–3984

Weatherston J, Percy JE (1978) Venoms of Coleoptera. In: Bettini S (ed) Arthropod venoms. Springer, Berlin, pp 511–554

Wheeler JW, Happ GM, Araujo J, Pasteels JW (1972) γ-Dodecalactone from rove beetles. Tetrahedron Lett 13:4635–4638

Will KW, Attygalle AB, Herath K (2000) New defensive chemical data for ground beetles (Coleoptera: Carabidae): interpretations in a phylogenetic framework. Biol J Linn Soc 71:459–481

Wu X, Buden DW, Attygalle AB (2007) Hydroquinones from defensive secretion of a giant Pacific millipede, *Acladocricus setigerus* (Diplopoda: Spirobolida). Chemoecology 17:131–138