J Chem Ecol (2009) 35:1302–1308
DOI 10.1007/s10886-009-9712-7

Differences in Defensive Volatiles of the Forked Fungus Beetle, *Bolitotherus cornutus*, Living on Two Species of Fungus

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Received: 22 August 2009 / Revised: 19 October 2009 / Accepted: 25 October 2009 / Published online: 20 November 2009
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Abstract Forked fungus beetles, *Bolitotherus cornutus*, feed, mate, and live on the brackets of several species of shelf fungus that grow on decaying logs. In response to the specific threat stimulus of mammalian breath, *B. cornutus* beetles produce a volatile defensive secretion. We tested beetles collected from different host fungi to determine whether defensive secretion blends varied with host type. Using solid phase microextraction and gas chromatography-mass spectrometry, we detected large amounts of the alkylated benzoquinones, methyl-*p*-benzoquinone (toluquinone) and ethyl-*p*-benzoquinone, and smaller quantities of *p*-benzoquinone, 3-methylphenol (*m*-cresol), 3-ethylphenol, 2-methylhydroquinone, and 2-ethylhydroquinone in secretions. Volatile composition did not differ between male and female beetles. Secretions did differ between beetles collected from two species of fungus, *Ganoderma applanatum* and *Fomes fomentarius,* with the relative amount of *p*-benzoquinone secreted being the most important factor. Other relationships among the volatile components are discussed.

Keywords Quinone · Solid phase microextraction · Phenol · Volatile · Defensive secretion · Tenebrionidae · Coleoptera

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Introduction

The quantity and identity of chemical defenses are known to vary with the diet of individual organisms when toxins are sequestered from the food source. This pattern holds across many taxa, ecological guilds, and classes of defensive compounds. Well known examples include the toxin blends of dart poison frogs that differ among individuals due to differences in the identity and frequency of particular arthropods in the diet (e.g., Saporito et al. 2009), the cardenolide content of individual monarch butterflies that depends on the toxicity of host plants on which larvae feed (e.g., Brower et al. 1982), as well as diet-based variations in the defensive secretions of the lubber grasshopper (Hatle and Spring 1998; Whitman et al. 1992). However, dietary influence is less well-documented among arthropods that biosynthesize (autogenous) defensive secretions from simple starting materials (Triponez et al. 2007). Variations with age, sex, season, and time for secretion regeneration all may affect the composition of these secretions (Blum 1981; Hill and Tschinkel 1985; Unruh et al. 1998).

The forked fungus beetle, *Bolitotherus cornutus* (Panzer) (Coleoptera: Tenebrionidae), is a nocturnal tenebrionid that lives, feeds, and breeds on the fruiting bodies ("brackets") of polypore fungi that grow on dead wood (Liles 1956). A given log produces brackets of only a single species of fungus. *Bolitotherus cornutus* populations are subdivided among logs within a forest, and adults rarely migrate from one log to another (Whitlock 1992). Beetles move mostly among brackets on the same log (Heatwole and Heatwole 1968), and thus normally experience a single species of fungus throughout their lives. Across their range in Northeastern North America, *B. cornutus* exploit three species of polypores—*Ganoderma applanatum*, *Ganoderma*

tsugae, and *Fomes fomentarius*—each of which may be found within the same local area of forest.

Bolitotherus cornutus' primary defensive response is a death feint (Liles 1956), but, like many other tenebrionid beetles, it can produce volatile defensive chemical secretions (Tschinkel 1975a). The defensive secretions are stored in eversible glands at the tip of the abdomen (Tschinkel 1975b). When the beetle is disturbed, the glands are everted and stroked rapidly with the hindlegs, thus spreading the secretion (Conner et al. 1985). Although other species of tenebrionids secrete or spray defensive chemicals in response to attack by other insects (Peschke and Eisner 1987), *B. cornutus* reliably produces defensive secretions only in response to the warmth, humidity, and pulse—but not to the carbon dioxide content—of mammalian breath (Conner et al. 1985). Even mechanical injury by using artificial jaws does not usually result in eversion of the glands (Conner et al. 1985).

Early investigation of the chemical composition of *B. cornutus* defensive secretions was accomplished by directly "milking" the glands of the beetles and analyzing the product with packed-column gas chromatography (Tschinkel 1975a). With this older instrumentation, two large peaks were observed and identified as methyl-*p*-benzoquinone and ethyl-*p*-benzoquinone. Although other quinones and alkenes were detected in other tenebrionids, they were not detected in *B. cornutus* (Tschinkel 1975a). Solid phase microextraction, in combination with capillary gas chromatography-mass spectrometry (SPME-GC-MS) now provides an improved method for collecting, separating, and detecting volatile species. Recent applications of this technique to the detection of the quinone secretions of beetles include analysis of the defensive secretions of the red flour beetle, *Tribolium castaneum* (Herbst) (Villaverde et al. 2007) by using a carboxen/polydimethylsiloxane fiber, and analysis of the sex pheromones of a desert tenebrionid beetle by using a polydimethylsiloxane fiber (Geiselhardt et al. 2008).

Fieldwork on *B. cornutus* behavior suggested that beetles found on different species of fungus have slightly different scents to human observers. By using SPME-GC-MS, we compared the volatile secretions of male and female beetles collected from two species of fungus, *Ganoderma applanatum* and *Fomes fomentarius*, in the southern Appalachian Mountains.

Methods and Materials

Beetle Collection and Maintenance Forked fungus beetles were wild-caught as adults in the immediate vicinity of Mountain Lake Biology Station in Giles County, Virginia, USA in early August. Specimens were collected during the day from the surfaces of fungus brackets. They were placed immediately in containers that contained a fungal fruiting body harvested from that particular log, and shipped live to the laboratory for chemical analysis. Twelve individuals were collected from 6 colonies of *Ganoderma applanatum*, and 6 individuals from 6 colonies of *Fomes fomentarius* within an area of 0.05 ha. The sex of adults was determined easily by the large horns and ventral hair patches present only on males (Liles 1956). Analysis commenced 2 d after beetles arrived in the laboratory. The majority of beetles was analyzed within 1 wk of arrival; three beetles were not analyzed until over 1 wk later.

Chemicals Standards of *p*-benzoquinone (CAS 106-51-4), methyl-*p*-benzoquinone (CAS 553-97-9), 3-methylphenol (CAS 108-39-4), 3-ethylphenol (CAS 620-17-7), 2-methylhydroquinone (CAS 95-71-6), and 4-ethylresorcinol (CAS 2896-60-8) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Instrumentation A Varian (Walnut Creek, CA) Saturn 2100T gas chromatography—mass spectrometer (GC-MS), equipped with a FactorFour VF-5ms column (5% phenyl-methyl, 30 m, 0.25 mm i.d., 0.25-μm, Varian, Walnut Creek, CA, USA) was used for the analysis**.** Two cm dual-layered solid phase microextraction (SPME) fibers coated with highly cross-linked 50/30-μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) were purchased from Supelco (Bellefonte, PA, USA). Before use, fibers were cleaned by leaving them in the GC-MS injector port at 250°C for 30 min.

Sample Collection All sampling was done at room temperature by using headspace solid phase microextraction (HS-SPME). The amount of a compound collected by HS-SPME depends on both its gas-phase concentration and its affinity to the fiber (Zhang and Pawliszyn 1993); however, the maintenance of constant sampling conditions allows comparisons among samples. Before each run, a beetle was placed gently into a clean 10 ml glass vial and allowed to settle for several minutes. Then, by using a straw, human breath was blown onto the beetle for 5 sec. Gland eversion was observed in all cases. The vial was sealed immediately with a septum cap, and the SPME fiber was pierced through and exposed. After 3 min of collection, the fiber was removed and inserted into the GC/MS injection port, where it was desorbed for 30 sec at 250°C. In between runs, the SPME fiber was cleaned for 90 sec at 250°C to ensure that there was no carry-over from one beetle to the next.

A baseline measurement was performed each day by following the above procedure with an empty vial. Similarly, runs were performed by following the same protocol with a beetle present, but without exposure to breath.

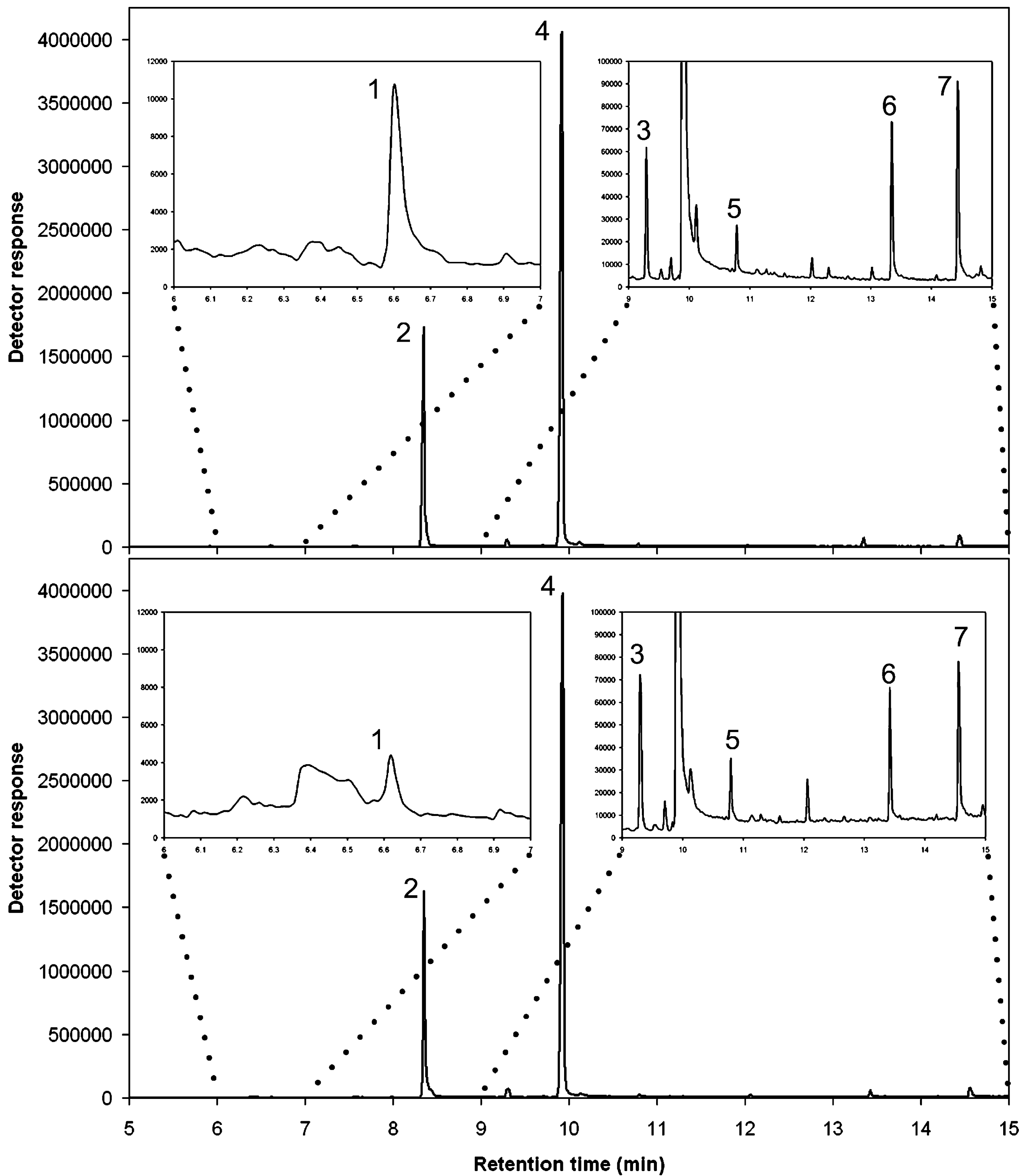


Fig. 1 Gas chromatogram of volatiles collected with solid phase microextraction from (*top*): a male *Bolitotherus cornutus* beetle collected from *Fomes fomentarius*; (*bottom*): a female *Bolitotherus cornutus* beetle collected from *Ganoderma applanatum.* 1: *p*-benzoquinone; 2: methyl-*p*-benzoquinone; 3: 3-methylphenol; 4: ethyl-*p*-benzoquinone; 5: 3-ethylphenol; 6: 2-methylhydroquinone; 7: 2-ethylhydroquinone. The scales for each portion of the graph are identical in the top and bottom pane

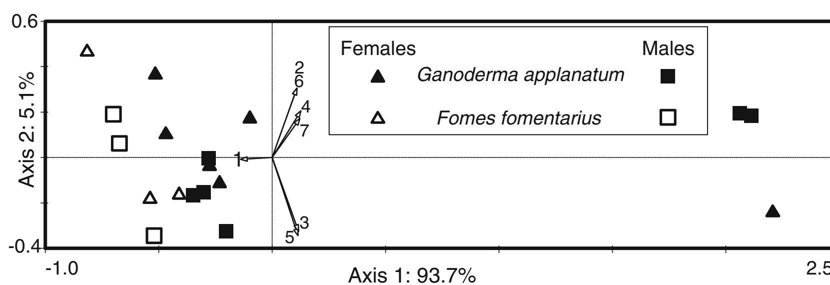


Fig. 2 Principal component analysis of centered log ratio transformed relative peak areas. 1: *p*-benzoquinone; 2: methyl-*p*-benzoquinone; 3: 3-methylphenol; 4: ethyl-*p*-benzoquinone; 5: 3-ethylphenol; 6: 2-methylhydroquinone; 7: 2-ethylhydroquinone

Gas chromatography-mass spectrometry A 20:1 split injection (column flow, He = 1.2 ml/min) into an injection port held at 250°C was used. Temperature programming: hold at 50°C for 3 min, ramp to 250°C at 10°C/min, and hold for 2 min.

With the exception of ethyl-*p*-benzoquinone and 2-ethylhydroquinone, all volatiles were identified by comparison of mass spectra and retention times with those of standards. Ethyl-*p*-benzoquinone (CAS 4754-26-1) was positively identified from its mass spectrum (NIST database), as well as by reference to prior identification of high concentrations of it in the defensive secretions of *B. cornutus* (Tschinkel 1975a). 2-ethylhydroquinone (CAS 2349-70-4) was identified tentatively by comparison of the mass spectrum to published data (Wahrendorf and Wink 2006), and by comparison of the retention time and mass spectrum to that of its structural isomer (with hydroxyl substituted at a different position on the ring), 4-ethylresorcinol.

Data Analysis As different beetles produced different absolute amounts of volatiles (statistically determined to be unconnected to sex or food source), a relative peak area was computed for each peak by dividing its area by the total area of the seven major, identified peaks for that beetle. Ordination analysis thus was done on fractional compositions that sum to unity, and for which the proportions of the different chemical compounds are not independent within a sample. Accordingly, a centered log ratio transformation (Aitchison 1984; Pawlosky-Glahn and Egozcue 2006) was employed prior to principal components analysis and its canonical equivalent, redundancy analysis, performed using CANOCO 4.5 (ter Braak and Smilauer 2002). In the case where a component was not detected, “0.00000001” was used in place of “0” as the relative peak area for that component in the calculation of centered log ratios (Aitchison and Egozcue 2005).

Results

The two major components of the beetles’ volatile secretions were methyl-*p*-benzoquinone and ethyl-*p*-benzoquinone. However, smaller amounts of *p*-benzoquinone, 3-methylphenol, 3-ethylphenol, 2-methylhydroquinone, and 2-ethylhydroquinone also were detected (Fig. 1).

By using the chemical composition of the beetles’ volatile defensive secretions, it is possible to differentiate beetles collected from *G. applanatum* from those collected from *F. fomentarius* (Fig. 2). The most important factor was the level of *p*-benzoquinone expressed. Three beetles collected from *G. applanatum* did not produce any detectable *p*-benzoquinone, and points for these beetles appear at the far right of axis 1 in Fig. 2. The points could, through their leverage, have a distorting or compressing effect on the rest of the ordination, thus inflating the effect of the *p*-benzoquinone. However, separation of beetles based on their fungal diet also was evident when the three points with high leverage were removed (Fig. 3). When fungal food source was used to constrain axis 1 of a redundancy analysis, the constrained axis explained 18.7% of the variance among beetles, and Monte Carlo permutations showed this axis to be significant (P=0.032). With the three beetles with no detectable *p*-benzoquinone excluded, the constrained axis was responsible for 23.8% of the

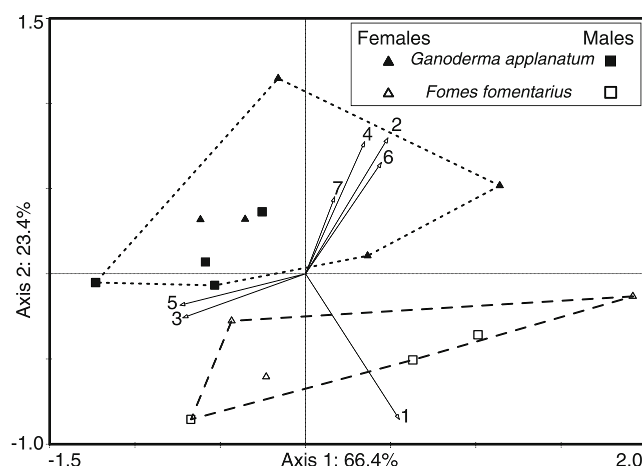


Fig. 3 Principal component analysis of centered log ratio transformed relative peak areas of defensive secretion of *Bolitotherus cornutus*, excluding three individiuals collected from *Ganoderma applanatum* that produced no detectable *p*-benzoquinone. 1: *p*-benzoquinone; 2: methyl-*p*-benzoquinone; 3: 3-methylphenol; 4: ethyl-*p*-benzoquinone; 5: 3-ethylphenol; 6: 2-methylhydroquinone; 7: 2-ethylhydroquinone

Table 1 Chemical composition of volatile secretions of male and female forked fungus beetles collected from two species of fungus. Values are given as mean relative peak areas, expressed as a percent, with associated standard errors

Food		N	*p*-benzoquinone	Methyl-*p*-benzoquinone	3-methylphenol	Ethyl-*p*-benzoquinone	3-ethylphenol	2-methyl-hydroquinone	2-ethyl-hydroquinone
Ganoderma applanatum	Female	6	0.03±0.01	29±2	1.4±0.4	67±2	0.5±0.2	0.8±0.2	1.1±0.2
	Male	6	0.03±0.01	23±2	1.8±0.4	73±2	0.6±0.2	0.9±0.1	1.3±0.2
	All	12	0.033±0.007	26±2	1.6±0.3	70±2	0.5±0.1	0.9±0.1	1.2±0.1
Fomes fomentarius	Female	3	0.16±0.03	28±2	1.9±0.8	67±2	0.5±0.2	1.2±0.2	1.7±0.3
	Male	3	0.19±0.01	26±1	2±1	68.3±0.3	0.5±0.3	1.1±0.1	1.63±0.06
	All	6	0.18±0.02	27±1	1.9±0.6	68±1	0.5±0.2	1.2±0.1	1.7±0.1

variance among beetles, with P=0.028. Whether or not the three beetles with no *p*-benzoquinone were included, there was no evidence from the ordinations of differences in volatiles associated with beetle sex.

Although *p*-benzoquinone was a minor component of the detected volatile species, beetles that were collected from *F. fomentarius* produced, proportionally, a much greater amount than those that were collected from *G. applanatum* (Table 1). The lowest producing *F. fomentarius*-fed beetle still produced 1.4 times as much *p*-benzoquinone as the highest producing *G. applanatum*-fed beetle (as a proportion of the total volatiles detected for the beetle). The relative peak area for *p*-benzoquinone was significantly different for beetles collected from the two fungal food sources (Two sample t test: t=8.94, *d.f.* = 16, P<0.001).

Several significant relationships were observed among the components. As seen in Fig. 4, the relative peak areas of 3-methylphenol and 3-ethylphenol are positively linearly correlated (r=0.95, N=18, Bonferroni-corrected P<0.001), and methyl-*p*-benzoquinone and ethyl-*p*-benzoquinone are negatively linearly correlated (r=−0.94, N=18, P<0.001). 2-Methylhydroquinone and 2-ethylhydroquinone also have a positive association, although the trend is not as clear as in the other two examples (r=0.71, N=18, P=0.02). No significant correlation was observed between the phenols and the quinones, or between *p*-benzoquinone and any other compound.

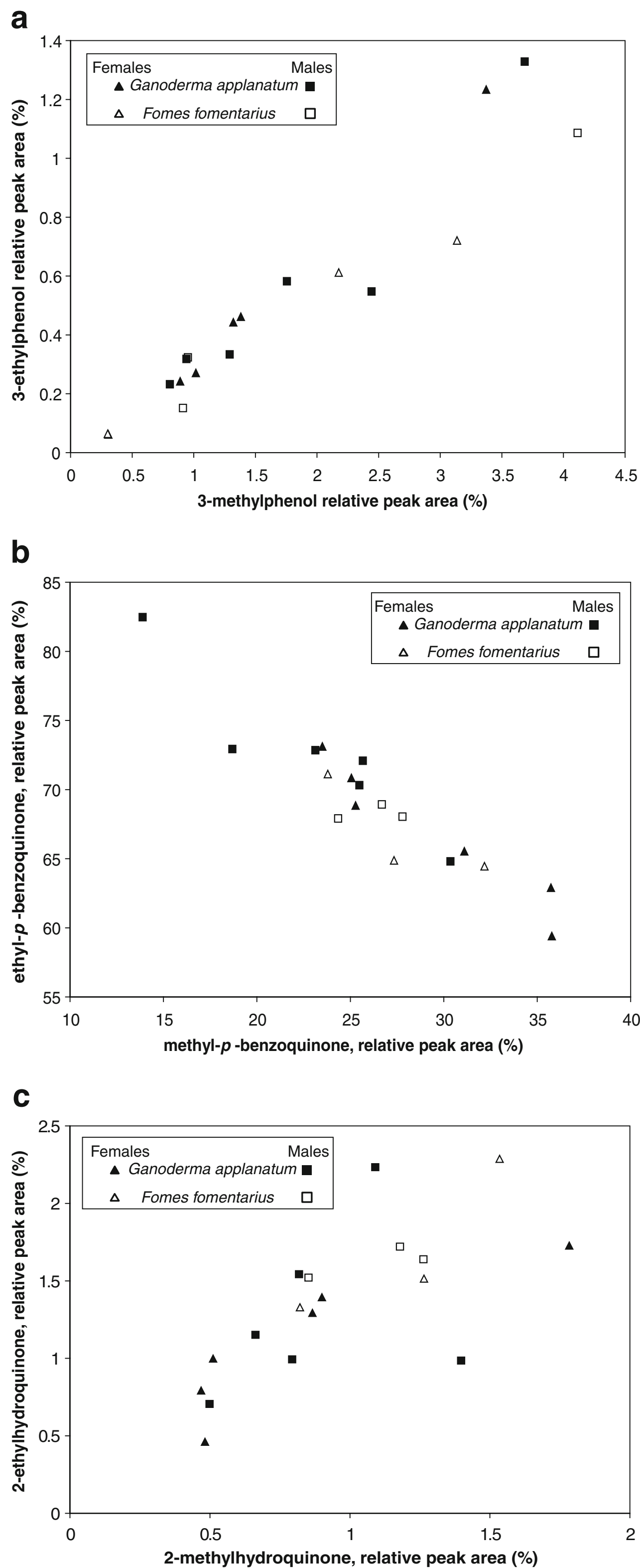
Discussion

Quinones are common in the defensive secretions of tenebrionid beetles (Tschinkel 1975a). Both methyl-*p*-benzoquinone and ethyl-*p*-benzoquinone previously were detected in the defensive secretions of *B. cornutus*, but the unalkylated *p*-benzoquinone was not (Tschinkel 1975a). Indeed, the lack of *p*-benzoquinone was seen as a distinguishing feature for *B. cornutus* among other tenebrionids that secrete quinones but not hydrocarbons (Tschinkel 1975a). We showed here that *p*-benzoquinone is produced, albeit in very small quantities, and that differences in diet and habitat may influence levels of this compound.

At first glance, it is surprising that *p*-benzoquinone has a much greater differentiating effect than similarly structured alkylated benzoquinones. However, this may be due to the presence of different biosynthetic pathways for the creation of alkylated benzoquinones (Meinwald et al. 1966). In *Eleodes longicollis* Lec., another tenebrionid beetle, Meinwald et al. (1966) demonstrated that *p*-benzoquinone was synthesized using the aromatic ring from either tyrosine or phenylalanine, whereas the alkylated benzoquinones were derived from acetate units. Therefore, differences in food sources may affect one pathway but not the other. *G. applanatum* fungus might have less bioavailable tyrosine or phenylalanine than *F. fomentarius*. Interestingly, some food source preferences have been observed in controlled observations of *B. cornutus* (Heatwole and Heatwole 1968), with *G. applanatum* being less preferred when another fungus species was available (*F. fomentarius* was not tested).

The three beetles collected from *G. applanatum* that did not produce any detectable *p*-benzoquinone were those that were sampled over a week later than the other beetles; perhaps some variation in living conditions caused the difference. However, these extremes of *p*-benzoquinone concentration did not enhance the separation between beetles collected from the two species of fungus, despite *p*-benzoquinone being the major distinguishing species between the two groups. In all other cases, a mixture of male and female beetles collected from *G. applanatum* and *F. fomentarius* were sampled on the same day.

3-Methylphenol (Tschinkel 1969; Attygalle et al. 1991; Villaverde et al. 2007; Geiselhardt et al. 2009;), 3-ethylphenol (Tschinkel 1969; Geiselhardt et al. 2009), 2-methylhydroquinone (Happ 1968; Wahrendorf and Wink 2006; Villaverde et al. 2007; Geiselhardt et al. 2009), and 2-ethylhydroquinone (Happ 1968; Hodges et al. 1996; Wahrendorf and Wink 2006; Geiselhardt et al. 2009) have all been detected in the defensive secretions of other tenebrionid beetles, although quinones are more ubiquitous



◀ **Fig. 4** Correlations between volatile components (relative peak areas, in percent) found for male and female *Bolitotherus cornutus* beetles collected from *Ganoderma applanatum* or *Fomes fomentarius*. **A** 3-ethylphenol as a function of 3-methylphenol. **B** ethyl-*p*-benzoquinone as a function of methyl-*p*-benzoquinone. **C** 2-ethylhydroquinone as a function of 2-methylhydroquinone

in tenebrionids. The presence of all of these chemical compounds emphasizes the likelihood that the alkylated benzoquinones found in *B. cornutus* are synthesized from acetate units.

3-Methylphenol, 2-methylhydroquinone, and methyl-*p*-benzoquinone are thought to be sequential members of a biosynthetic pathway that originates with 6-methylsalicylic acid (Gnanasunderam et al. 1984), itself formed from acetate (or malonate) units (Birch et al. 1955). Similarly, 3-ethylphenol, 2-ethylhydroquinone, and ethyl-*p*-benzoquinone are likely derived from 6-ethylsalicylic acid (Gnanasunderam et al. 1984), which differs in its synthesis by the use of a propionate unit in place of an acetate (or malonate) unit, as evidenced by isotope tracing experiments on ethyl-*p*-benzoquinone (Meinwald et al. 1966). We did not detect any salicylic acids in the defensive secretions of *B. cornutus*, but their methyl esters have been found in the secretions of another tenebrionid beetle (Gnanasunderam et al. 1984).

In view of this pathway, one way to explain the positive correlation between the relative peak areas of the phenols (or hydroquinones) is by attributing their relationship to a coupled series of biochemical reactions in which the formation of one leads to favorable conditions for the production of the other. The negative correlation observed between the relative peak areas of the alkylated quinones might be explained by a competitive inhibition of the oxidase used to form quinones from hydroquinones (Happ 1968). It is interesting that correlations were seen only between methyl and ethyl analogs at each structural level; no correlations were found between members of a biosynthetic pathway. The lack of correlation between either alkylated benzoquinone and *p*-benzoquinone further emphasizes its different biosynthetic origins.

No significant differences were seen between the sexes. Therefore, although both phenols (Geiselhardt et al. 2008) and quinones (Ruther et al. 2001; Geiselhardt et al. 2008) are used as sex pheromones in other beetle species, this does not appear to be the case for *B. cornutus*. This is not unexpected, as the volatiles are emitted in response to a specific threat stimulus.

The ecological effect of the observed differences in chemical blends is unclear. Although, in comparison to *p*-benzoquinone, the alkylated benzoquinones have been shown to have greater repellant effects against ants and induce greater topical irritation in cockroaches (Peschke and Eisner 1987). *Bolitotherus cornutus* emits these compounds only in response to detected threats from mammalian predators. Some human observers can detect qualitative odor differences in the blends, but do not notice differences in overall repugnance, although *p*-benzoquinone is known to be a powerful irritant (Eisner and Aneshansley

1999). Further experiments with isolated *p*-benzoquinone or blends of quinones that vary in relative ratios of *p*-benzoquinone will elucidate the importance of dietary differences to anti-predator function.

Acknowledgements We thank Neil J. Holliday for statistics advice and Swarthmore College for support of this research. Mountain Lake Biological Station provided support for the field component. Corlett Wood, Eileen Butterfield, and Mollie Barnard assisted with fieldwork, and Lisa Schumacher assisted with laboratory analysis.

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