The Science of Nature (2020) 107: 26
https://doi.org/10.1007/s00114-020-01683-0

ORIGINAL PAPER

Biosynthetic origin of benzoquinones in the explosive discharge of the bombardier beetle *Brachinus elongatulus*

Athula B. Attygalle[1] · Sihang Xu[1] · Wendy Moore[2] · Reilly McManus[2] · Aman Gill[3] · Kipling Will[3]

Received: 29 February 2020 / Revised: 5 May 2020 / Accepted: 18 May 2020 / Published online: 16 June 2020

Abstract

Bombardier beetles are well-known for their remarkable defensive mechanism. Their defensive apparatus consists of two compartments known as the reservoir and the reaction chamber. When challenged, muscles surrounding the reservoir contract sending chemical precursors into the reaction chamber where they mix with enzymes resulting in an explosive discharge of a hot noxious chemical spray containing two major quinones: 1,4-benzoquinone and 2-methyl-1,4-benzoquinone (toluquinone). Previously, it has been speculated that the biosynthesis of all benzoquinones originates from one core precursor, 1,4-hydroquinone. Careful ligation of the base of the reservoir chamber enabled us to prevent the explosive reaction and sample untransformed reservoir fluid, which showed that it accumulates significant quantities of 1,4-hydroquinone and 2-methyl-1,4-hydroquinone. We investigated the biosynthetic mechanisms leading to quinone formation by injecting or feeding *Brachinus elongatulus* beetles with stable-isotope-labeled precursors. Chemical analysis of defensive secretion samples obtained from 1,4-hydroquinone-d_6-administered beetles demonstrated that it underwent conversion specifically to 1,4-benzoquinone. Analogously, results from *m*-cresol-d_8 injected or fed beetles confirmed that *m*-cresol is metabolized to 2-methyl-1,4-hydroquinone, which is then oxidized to 2-methyl-1,4-benzoquinone in the hot spray. Our results refute the previous claim that 1,4-hydroquinone is the precursor of all substituted benzoquinones in bombardier beetles and reveal that they are biosynthetic products of two independent pathways. Most likely, the aforementioned biosynthetic channel of hydroxylation of appropriate phenolic precursors and subsequent oxidation is not restricted to bombardier beetles; it could well be a general pathway that leads to the formation of all congeners of benzoquinones, one of the most widely distributed groups of defensive compounds in arthropods.

Keywords Benzoquinone · Biosynthesis · Bombardier beetles · *Brachinus elongatulus* · Defensive secretions

Introduction

Carabid beetles (Carabidae) are well known for using chemical weapons against their enemies (Eisner 1958; Dazzini-Valcurone and Pavan 1980; Dettner 1985; Will et al. 2000). Among the defensive behaviors exhibited by carabids, the most fascinating is the explosive chemical spraying of beetles belonging to the subfamilies Brachininae and Paussinae (Schildknecht 1957; Schildknecht and Koob 1969; Schildknecht et al. 1970). Commonly known as "bombardier beetles," these beetles have the remarkable ability to explosively discharge with a popping sound a hot noxious chemical spray containing benzoquinones from glandular openings near their abdominal tip (Arndt et al. 2015). In fact, this ejection system has been considered to show fundamental similarity to the pulse jet propulsion mechanism of the German V-1 "buzz" bomb of World War II (Eisner and Aneshansley 1982; Dean et al. 1990).

Communicated by: John A. Byers

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00114-020-01683-0) contains supplementary material, which is available to authorized users.

✉ Athula B. Attygalle
athula.attygalle@stevens.edu

[1] Department of Chemistry and Chemical Biology, Stevens Institute of Technology, Hoboken, NJ 07030, USA

[2] Department of Entomology, University of Arizona, Tucson, AZ 85721, USA

[3] Department of Environmental Science, Policy and Management, University of California, Berkeley, CA 94720, USA

Although the defensive secretions of many species of the genus *Brachinus* have been investigated, that of *Brachinus elongatulus* has not been analyzed previously. However, the morphology of the defensive glands of *Brachinus elongatulus* Chaudoir and other bombardier beetles have been described in a series of recent studies (Arndt et al. 2015; Di Giulio et al. 2015; McManus et al. 2018). Similar to all members of the beetle suborder Adephaga (Ober and Maddison 2008; Arndt et al. 2017), bombardiers possess a pair of homologous pygidial glands composed of secretory cells, a reservoir and efferent duct (Balestrazzi et al. 1985). Over the course of evolution of this system (Moore and Wallbank 1968; Maddison et al. 2009), the bombardiers acquired a highly sclerotized chamber known as the reaction chamber, which is separated from the relatively larger reservoir compartment by a one-way valve (Arndt et al. 2015). Bombardiers do not store the final components used in the spray. Instead, they produce the explosive mixture on demand, at the moment of ejection. It has been presumed that the reservoir contains hydroquinones and hydrogen peroxide that function as the precursors for benzoquinone synthesis (Schnepf et al. 1969). When bombardier beetles are provoked, the muscles surrounding the reservoir contract, enabling the reservoir contents to pass through the one-way valve and enter the reaction chamber (Arndt et al. 2015; Di Giulio et al. 2015). The instant hydroquinones and hydrogen peroxide encounter the enzymes in the reaction chamber (Schildknecht and Holoubek 1961), an explosive exothermic reaction takes place producing a mixture of benzoquinones, molecular oxygen, and water, heated up to 100 °C (Schildknecht and Holoubek 1961; Schildknecht et al. 1968, 1970; Aneshansley et al. 1969).

Benzoquinones are not unique to bombardier beetles. In fact, benzoquinones are widely distributed in nature (Abraham et al. 2011; Thomson 1997). They have been reported from bacteria, plants, and many other arthropods (Blum 1987; Happ 1968; Chadha et al. 1961; Dettner 2010) including millipedes (Deml and Huth 2000; Wu et al. 2007; Sekulić et al. 2014; Shear 2015; Bodner et al. 2016) and harvestmen (Fieser and Ardao 1955; Eisner et al. 2004; Raspotnig et al. 2015; Rocha et al. 2013; Gnaspini and Cavalheiro 1998). Within insects, benzoquinones are widespread in cockroaches (Roth and Stay 1958; Nojima et al. 2005), earwigs (Eisner et al. 2000; Gasch et al. 2013), termites (Olagbemiro et al. 1988), and beetles of many families (Blum 1981; Dettner 1991). Apart from their defensive activity, benzoquinones may even act as sex pheromones in scarabaeoid beetles (Vuts et al. 2014).

Although many investigations have been carried out to identify the quinones in beetles, little attention has been given to the biosynthetic origin of these compounds (Happ 1968; Bradshaw 1985; Blum 1987; Morgan 2010). Many investigators have found biosynthetic studies of benzoquinones in arthropods to be challenging and less rewarding than one might hope. For example, in a very early study on the biosynthetic studies of simple benzoquinones in Jamaican millipedes, Dufey (1974) could not shed much light on the reaction pathways after 2 years of labor. On the other hand, many hypothetical pathways have been proposed with little or no experimental evidence to support these assumptions. For example, Deml and Huth (2000) speculated that both 1,4-benzoquinone (Fig. 1a) and 2-methyl-1,4-benzoquinone (toluquinone; Fig. 1b) originate from the same precursor 1,4-hydroquinone (Fig. 1c) (Shear 2015). However, in a very early study, Meinwald et al. (1966) indicated that in the beetle *Eleodes longicollis*, 1,4-benzoquinone is primarily made from the preformed rings of the aromatic amino acids such as tyrosine or phenylalanine, whereas substituted quinones originate from acetate by the polyketide pathway (Blum 1987). More recently, the biosynthesis of benzoquinones in harvestmen (Opiliones) has been demonstrated to follow a multistep pathway that involves condensation of acetate and propionate units (Raspotnig et al. 2015; Rocha et al. 2013). Moreover, Rocha et al. (2013) indicated that 2-ethyl-1,4-benzoquinone is biosynthesized by the harvestman *Magnispina neptunus* via 3-ethylphenol.

Thus, we predicted that benzoquinones in bombardier beetles might originate in an analogous manner, and the putative biosynthetic precursor of 2-methyl-1,4-benzoquinone (Fig. 1b) could be *m*-cresol. To test this hypothesis, we injected a solution of *m*-cresol-d_8 (Fig. 1e) in saline to the hemocoel of several individuals of *B. elongatulus* and subsequently analyzed the defensive spray. Moreover, we tested the long-held hypothesis that the immediate precursors of benzoquinones accumulate in the reservoir. To test this hypothesis, we ligated the base of the reservoir, isolating it from reaction chamber, to verify whether the reservoir contains an accumulation of hydroquinones, which are usually found only as trace components in the sprayed defensive secretion.

Materials and methods

Chemicals

m-Cresol-d_8 (Fig. 1e) and 1,4-hydroquinone-d_6 (Fig. 1f) were purchased from Cambridge Isotopes (Tewksbury, MA), or Sigma-Aldrich Chemical Co (St. Louis, MO). The stock solutions of the isotope-labeling compounds were made in saline (2 mg/100 μL) by dissolving 20 mg of each compound in 1.0 mL of saline solution.

Collection and handling of beetles and sample preparation

Brachinus elongatulus beetles were collected in Pima Co., AZ, and transferred to laboratory facilities at UC Berkeley, CA. Beetles were maintained on moist sphagnum, potting

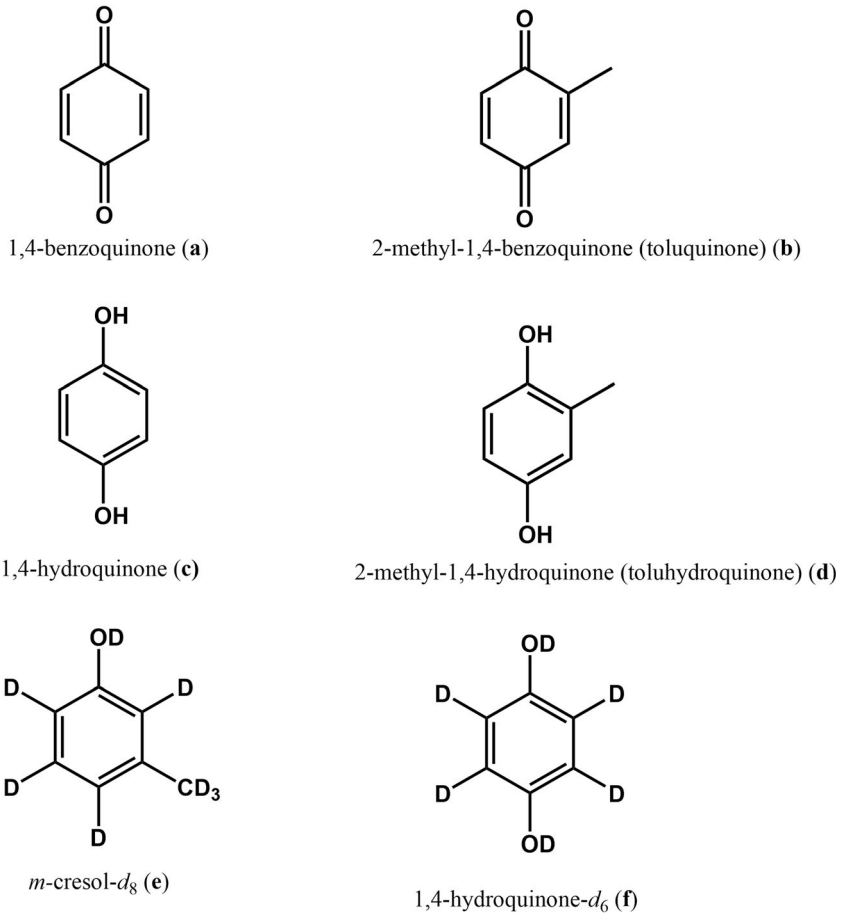

Fig. 1 Structures of chemicals used in this study

soil, and sand mix in an environmental chamber at a day/night regime of 13 h light/11 h dark, and day/night temperatures of 23 °C/16 °C. When not receiving any specific treatment, they were held in aggregation and fed dry, high-protein dog food (Blue Buffalo Co, Wilton, CT), banana, and chopped meal worms approximately once per week.

For feeding controls and treatments, the dry-dog food was ground and sifted. Particles larger than 0.5–1.0 mm were retained and used for feeding. Approximately 12.0 mg of the sifted dog food was placed in caps cut from 0.2-mL PCR tubes. For each feeding session, 1.5 μL of saline or *m*-cresol-d_8 in saline solution (20 mg/mL) was applied to the food placed in the caps immediately prior to presenting it to the beetle. In some cases, beetles were observed to feed immediately, but typically they feed at night and the food dish was left in the container with the beetle for two nights. Uneaten food and dishes were removed prior to the subsequent feedings. Starting on the day individual beetles were segregated, they were presented food on days 1, 3, 5, 8, 14, 21, and 24, up to the day of dissection or sampling. Altogether, 18 beetles were used for the feeding experiments (2 females, 1 male, and 15 sex-undetermined specimens).

Solutions were directly injected using a 5.0-μL borosilicate glass syringe with a 33-gauge, 12° bevel point needle. A single syringe and set of needles were dedicated to each treatment to prevent cross-contamination. The needle was inserted into the abdomen of the beetles in the membranous area between the 3rd and 4th ventrites. After injections, needles and syringes were cleaned with a series of washes using water, methanol, and hexane. By this procedure, *m*-cresol-d_8 in saline was injected into 23 beetles (1 female, 3.0 μL; 1 female, 3.5 μL; 2 females, 4.0 μL; 1 male, 2.0 μL; 18 sex-undetermined specimens, 5.0 μL each).

Samples of glandular products of beetles were obtained either by dissecting out the glands or by catching ejected spray on filter paper. Sprays were collected by placing a small strip of clean filter paper behind the beetle and stimulating it to spray by slightly pinching a leg with forceps. Filter paper samples were immediately placed in glass vials with solvent, according to the method of Eisner et al. (1977). Beetles were either chilled in a freezer as described below and held with a pair of forceps as they returned to room temperature, at which point they spray or were tethered as done by Aneshansley et al. (1969) and induced to spray.

Live beetles were carefully handled to prevent any discharges and placed in a 0 °C freezer for approximately 3 min to anesthetize and immobilize them. For dissections, an immobilized beetle was then placed on a pre-chilled watch

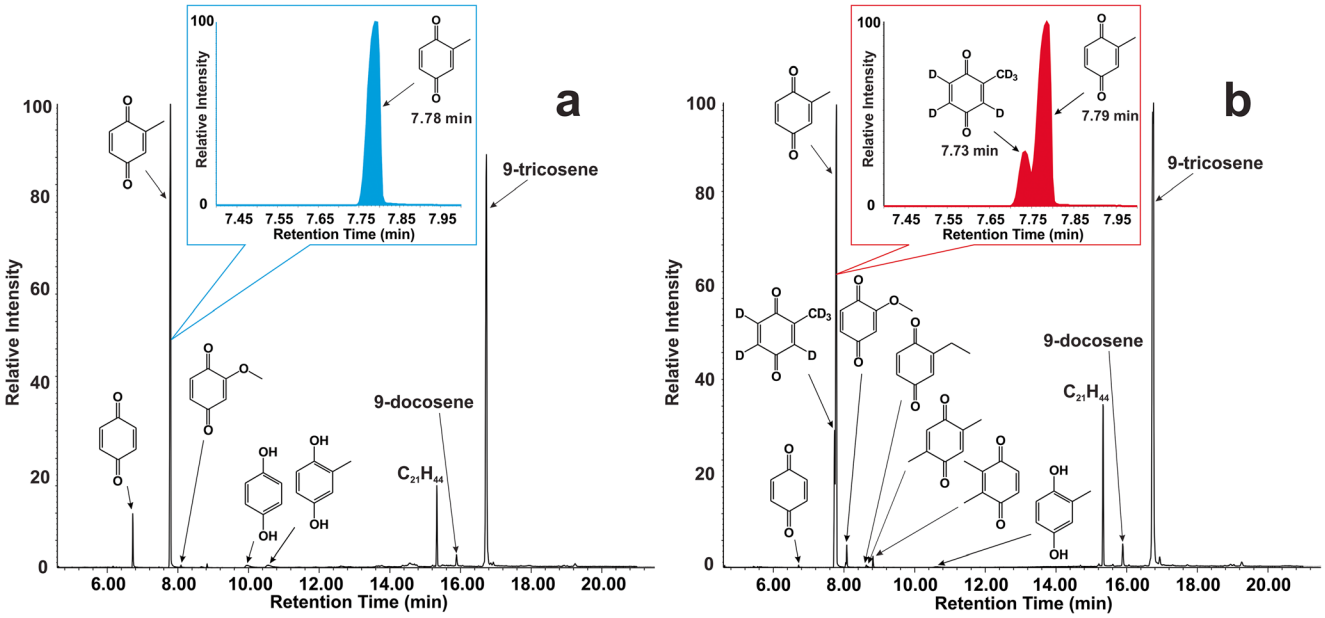

Fig. 2 Gas chromatograms obtained from GC-MS analysis of a hexane extract of defensive glands of *B. elongatulus* prepared 7 days after the injection of 5 μL of saline (**a**) or a solution of *m*-cresol-d_8 in saline (**b**). Insets show expansions of the 7.4–8.0 min region of the chromatograms. Analyses were performed on a fused-silica capillary column coated with DB-1

glass, and the abdomen was separated from the rest of the body. The abdomen was dissected further on a glass plate held at room temperature. The tergum was cut along the epipleura and lifted off to reveal the internal organs. A very small amount of distilled water was used to prevent the tissue from sticking to the forceps, and a drop of water was placed on the open abdomen to prevent it from drying during dissection. Any residual water on the dissected structures was wicked away with a small strip of clean filter paper prior to placing it into the solvent.

Either one or both of the left and right reservoirs and reaction chambers were excised as a whole gland complex or individually ligated and separated. Whole glands were removed by pulling the reaction chamber away from the tergite and lifting the entire reservoir and reaction chamber out of the abdomen and placing the whole system into a glass vial containing hexane (25–40 μL). The relative fullness of the reservoirs was evaluated, and if very full each whole gland was removed and placed in a vial. If a single beetle appeared to have relatively little fluid in both of the left and right reservoirs, both were pooled into a vial as one sample.

In order to sample the reservoir fluid without the inclusion of the reaction chamber contents, a separate reservoir-ligation dissection was performed. To ligate the reservoir, an overhand knot was tied in a single strand of human hair, and the loop of the knot was slipped over the reservoir while it was still in the abdomen. The knot was carefully cinched down around the base of the reservoir just above the reaction chamber. By gently pulling the hair, the reservoir was separated from the abdomen, and the reservoir was broken at its junction with the

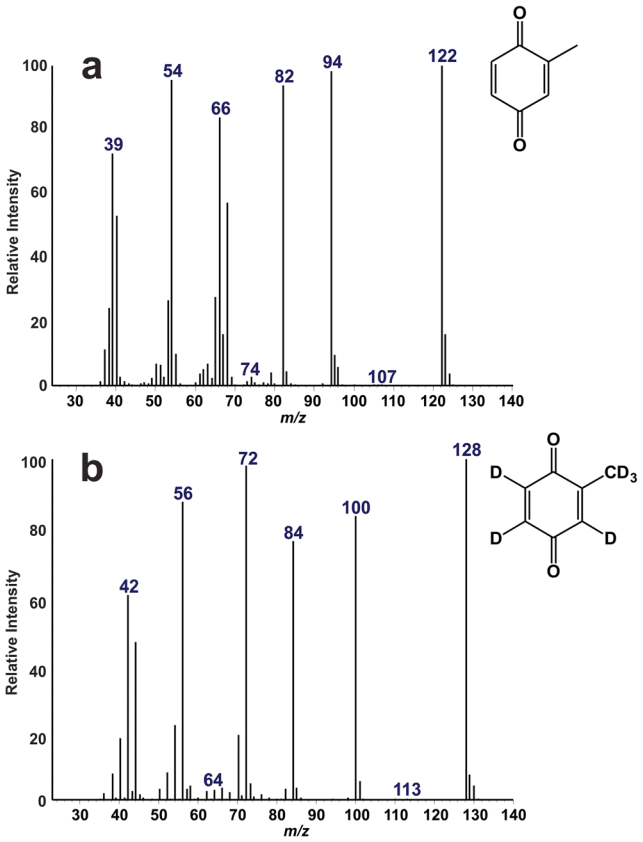

Fig. 3 Electron-ionization (70 eV) mass spectra corresponding to the 2-methyl-1,4-benzoquinone peak in Fig. 2b (**a**), and that for the deuteriated compound that eluted immediately before the 2-methyl-1,4-benzoquinone peak (**b**)

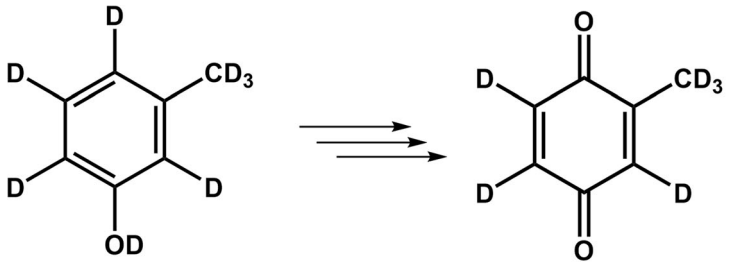

Fig. 4 Conversion of *m*-cresol-d_8 (**a**) to 2-methyl-1,4-benzoquinone-d_6 (**b**)

reaction chamber. In this way, the reservoir contents were held intact, and there were no apparent signs of the benzoquinone-forming reaction occurring (no color change). The strand of hair was trimmed near the knot, and the ligated reservoir was placed in a glass vial containing hexane (25–40 μL). Seven beetles used as controls received only the saline solution. Beetles fed with saline-treated food were then subjected to dissection of the whole gland (one specimen, sex was not determined), dissection of the ligated reservoir (one female and one male), or spray on filter paper (one female and one male). Beetles injected with 5.0 μL saline were subject to similar treatment.

Eleven beetles were treated with 1,4-hydroquinone-d_6 injections: three females (two injected with 5.0 μL, one injected with 3.5 μL), two males (injected with 5.0 μL), three beetles of undetermined sex (injected with 5.0 μL), and three females (injected with 5.0 μL and subsequently fed with dog food mixed with 5 μL of 1,4-hydroquinone-d_6). These beetles were then maintained individually and fed with untreated dog food on days 1, 3, and 5, and then dissected on days 7–9 and the glandular apparatus of each specimen was removed and extracted as above.

Samples, either whole gland dissections or filter papers containing sprayed secretions, were placed in hexane (25–40 μL) and shipped to Hoboken, NJ, in an insulated box chilled with Koolit-gel packs.

Chemical analysis

Samples were analyzed by GC-MS on an Agilent HP 6890 Gas Chromatograph (GC) fitted with a fused-silica capillary column coated with DB-1 (dimethylpolysiloxane, 30 m × 0.25 mm ID, film thickness 0.25 μm) linked to Agilent HP 5973 Mass Selective Detector (MSD). Samples were

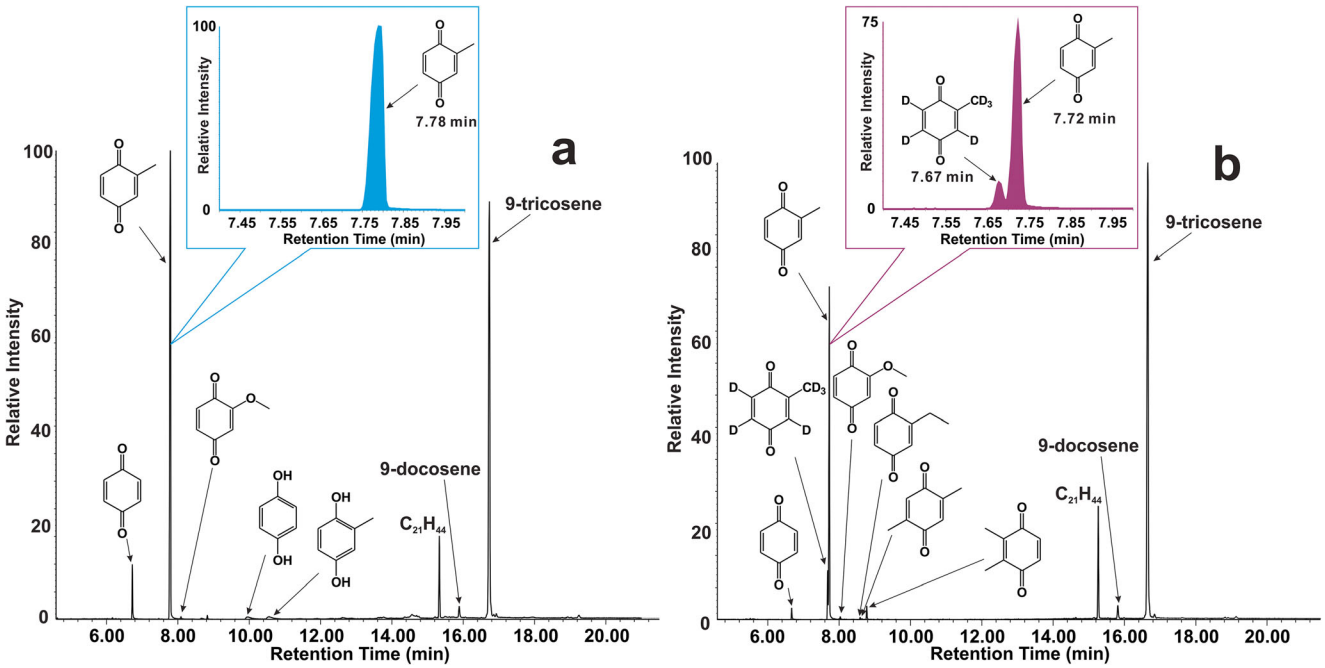

Fig. 5 Gas chromatograms obtained from GC-MS analysis of a hexane extract of defensive glands of *B. elongatulus* after injecting 5 μL of saline (**a**) or after feeding of laced food admixed with *m*-cresol-d_8 for about 3 weeks (**b**). Insets show expansions of the 7.4–8.0 min region of the chromatograms. Analysis were performed on a fused-silica capillary column coated with DB-1

introduced by splitless injection at 250 °C. The GC oven was kept initially at 40 °C for 2.00 min and raised at a rate of 20 °C/min to a final temperature of 280 °C and held for 7.5 min. Alternatively, some samples were analyzed on a Shimadzu QP5050 mass spectrometer linked to a Shimadzu GC-17A gas chromatograph (GC) installed with a DB-624 (6% cyanopropyl-phenyl, 94% dimethylpolysiloxane; film thickness 1.12 μm) coated 25 m × 0.20 mm ID capillary column. The oven temperature program was 100 °C initially and increased at a rate of 10 °C/min to a final temperature of 250 °C.

Derivatization with dimethyl disulfide

An aliquot of secretion extract (5 μL) was mixed with dimethyl disulfide (2 μL) in a reaction vial (100 μL). A solution of iodine in ether (5%, 10 μL) was added, and the mixture was kept overnight at room temperature. Afterward, the reaction mixture was decolorized with a minimum volume of aqueous sodium thiosulfate (5%). Products were extracted into hexane and examined by GC-MS on a DB-1 coated capillary column (Francis and Veland 1987; Buser et al. 1983). The initial oven temperature was 150 °C, which was immediately raised to 295 °C at a rate of 15 °C/min and held constant until all derivatives eluted.

Results

The analysis of an extract made from the defensive apparatus of *B. elongatulus* by GC-MS showed that the major component in the secretion is 2-methyl-1,4-benzoquinone (Fig. 2a).

A typical gas chromatogram recorded from control specimens showed two major peaks for 1,4-benzoquinone (Fig. 1a) and 2-methyl-1,4-benzoquinone (Fig. 1b), in addition to a few minor signals for methoxy-1,4-benzoquinone, ethyl-1,4-benzoquinone, 2,5-dimethyl-1,4-benzoquinone, 2,3-dimethyl-1,4-benzoquinone, and traces of 1,4-hydroquinone (Fig. 1c) and 2-methyl-1,4-hydroquinone (Fig. 1d) (Fig. 2a). All compounds were identified by comparing retention times and mass spectra with those of synthetic standards (see Supplementary Table S1 for analytical details). The hydrocarbon peaks were identified as heneicosane, 9-docosene, and 9-tricosene (Supplementary Figures S1 and S2). Compared with the gas chromatographic profiles generated from hexane extracts prepared from control specimens, those recorded from beetles injected with *m*-cresol-d_8 showed an additional chromatographic peak of significant intensity, which eluted immediately before the 2-methyl-1,4-benzoquinone peak. Typical profiles recorded from two beetles kept for 7 days before dissection are depicted in Fig. 2b (analytical details of all other samples and results are summarized in Supplementary Table S2).

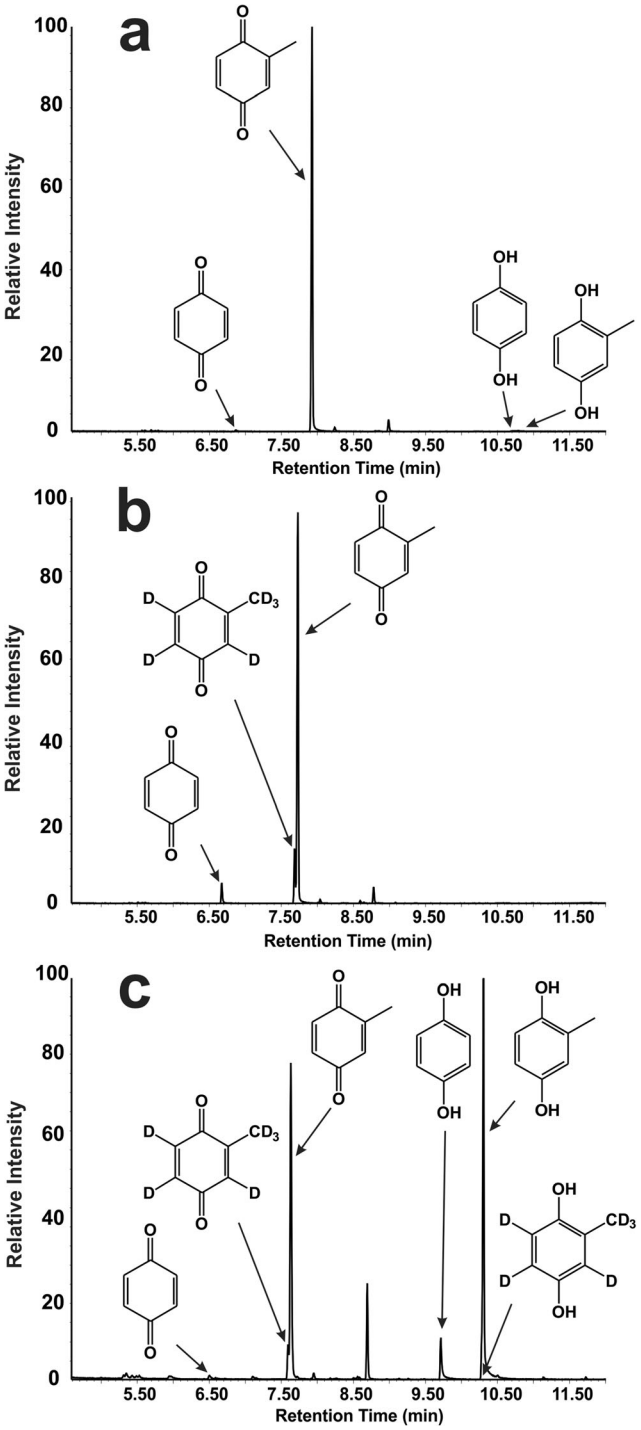

Fig. 6 Chromatograms obtained from GC-MS analysis of a hexane extract of defensive glands of a control *B. elongatulus* sample (**a**) and that of a *m*-cresol-d_8-fed specimen (**b**). **c** Chromatogram recorded from an extract of the reservoir fluid of a *m*-cresol-d_8-fed specimen that was prevented from mixing with the reaction chamber contents by ligation. Analysis were performed on a fused-silica capillary column coated with DB-1

The electron-ionization mass spectrum corresponding to the additional chromatographic peak that eluted immediately before the 2-methyl-1,4-benzoquinone peak showed

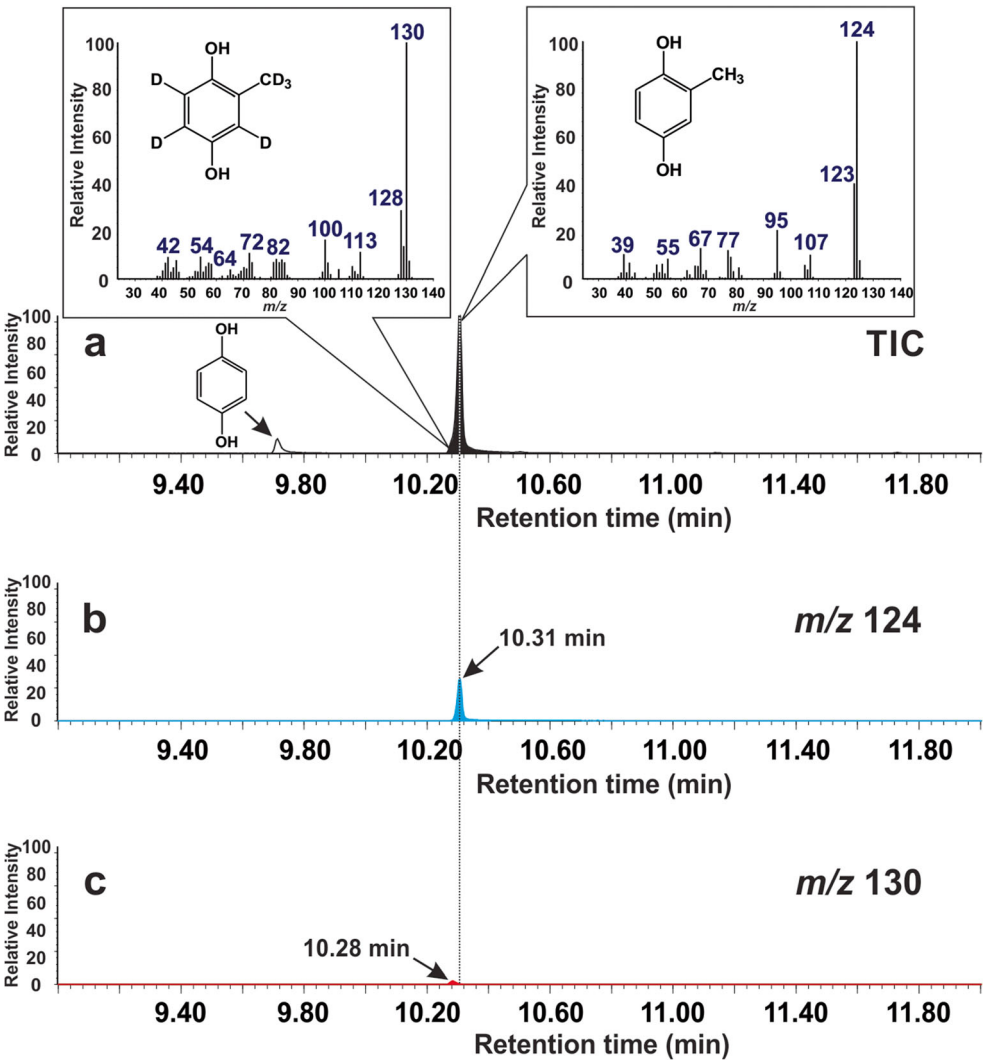

Fig. 7 An expansion of the 9.0- to 12.0-min region of the total-ion chromatogram depicted in Fig. 6c (**a**) and ion chromatograms extracted from acquired data for *m/z* 124 (**b**) and *m/z* 130 (**c**). Insets show background-subtracted mass spectra corresponding to two specific points of the chromatogram. Analysis were performed on a fused-silica capillary column coated with DB-1

a signal at *m/z* 128 for its molecular ion (Fig. 3b). On the basis of mass spectral interpretations provided by Beynon and Williams (1960), it was evident that of the eight deuterium atoms initially present in the *m*-cresol-d_8 (Fig. 4a) precursor compound, only six of them were incorporated into the product (Fig. 4b). This result conclusively showed that *B. elongatulus* beetles are able to convert *m*-cresol-d_8 (Fig. 4a (nominal mass 116 u), injected into the hemocoel), into 2-methyl-1,4-benzoquinone-d_6 (Fig. 4b; nominal mass 128 u) (Fig. 4).

Remarkably, *B. elongatulus* beetles were capable of converting *m*-cresol-d_8 (Fig. 4a; nominal mass 116 u) into 2-methyl-1,4-benzoquinone-d_6 (Fig. 4b; nominal mass 128 u) even when the precursor compound was only added to their food. In fact, incorporations were detected even after 24 h of feeding. Figure 5 b shows data recorded from a specimen that was continuously fed food mixed with the deuterium-labeled compound for about 3 weeks. Moreover, the deuterium labeling in *m*-cresol-d_8 (Fig. 1e) in both experiments (injection and feeding) was

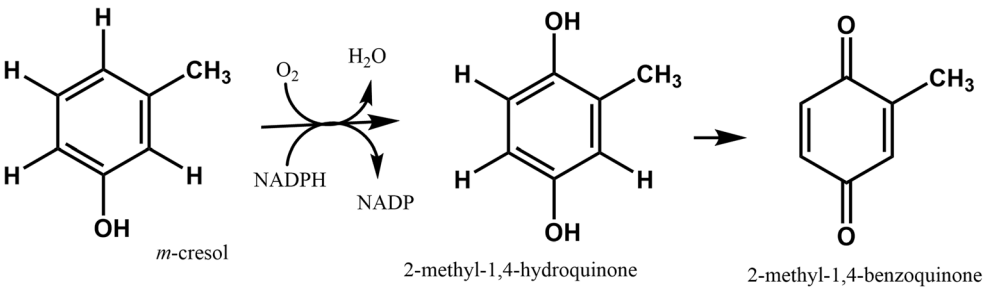

Fig. 8 Conversion of *m*-cresol to 2-methyl-1,4-benzoquinone via 2-methyl-1,4-hydroquinone

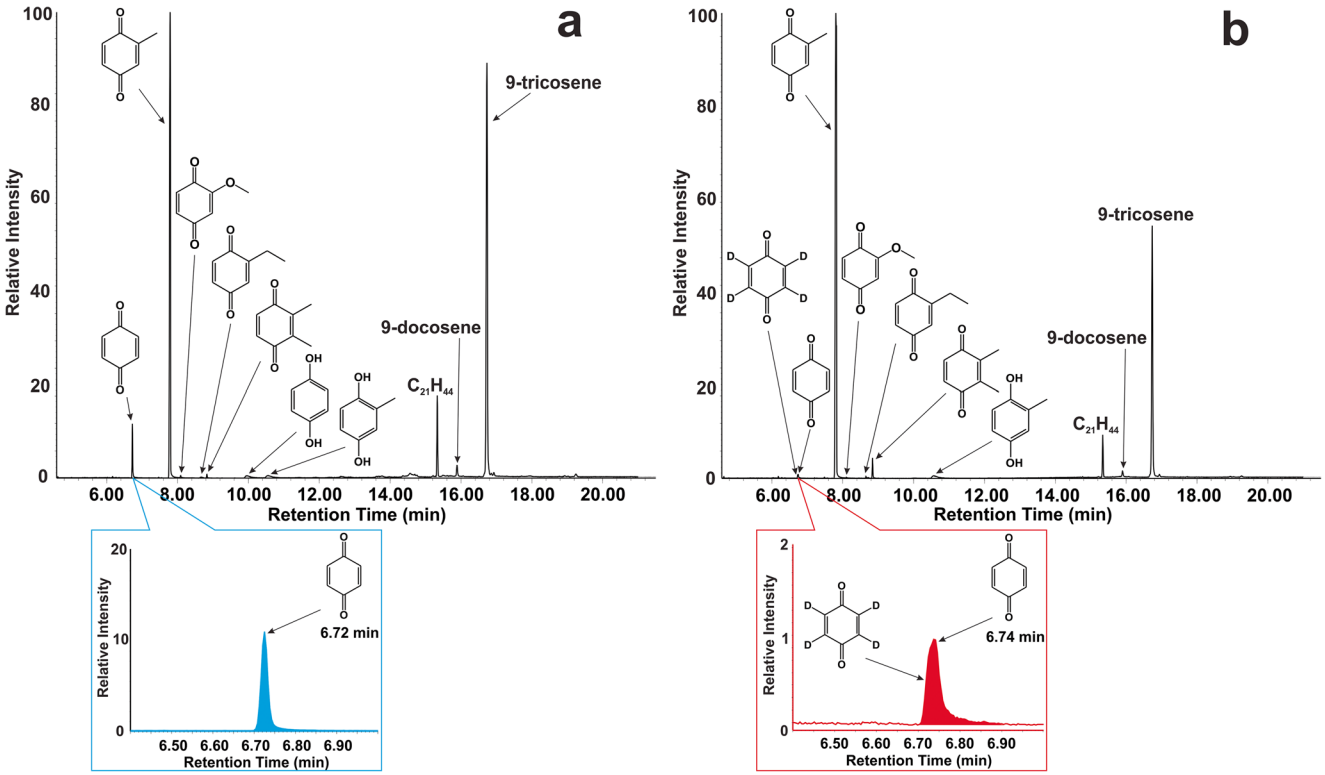

Fig. 9 Gas chromatograms obtained from GC-MS analysis of a hexane extract of defensive glands of *B. elongatulus* after injection of 5 μL of saline (**a**) or solution of 1,4-hydroquinone-d_6 in saline (**b**). Analysis were performed on a fused-silica capillary column coated with DB-1

incorporated specifically only to 2-methyl-1,4-benzoquinone (Fig. 1b) and not into 1,4-benzoquinone (Fig. 1a) or any other quinone present in the secretion in trace amounts.

Chromatograms recorded from extracts of the reservoir fluid from ligated reservoirs showed pronounced peaks for the two hydroquinones (1,4-hydroquinone (Fig. 1c) and 2-methyl-1,4-hydroquinone (Fig. 1d)), which are usually found only as trace components in the sprayed defensive secretion (Supplementary Figure S3). For example, in the chromatograms recorded from extracts of the whole glandular complex, the hydroquinone peaks were negligibly small (Figs. 2a and 5a). Evidently, our ligation technique reduces the formation of benzoquinones significantly; however, the technique does not provide a hermetic seal and prevent their formation completely (Fig. 6c).

We hypothesized that these hydroquinones are the immediate precursors of the benzoquinones. In order to test our hypothesis, we extracted ligated reservoirs of *m*-cresol-d_8-fed beetles. The chromatogram recorded from these samples showed a more pronounced peak for 2-methyl-1,4-hydroquinone (Fig. 6c) and also a small peak for the presence of 2-methyl-1,4-hydroquinone-d_6 (Fig. 1f). The formation of 2-methyl-1,4-hydroquinone-d_6 was confirmed by extracting an ion-intensity profile for the *m/z* 130 ion (Fig. 7c). The retention time of the 2-methyl-1,4-hydroquinone-d_6 peak was slightly shorter than that of the 2-methyl-1,4-hydroquinone peaks as that expected from a deuteriated analog. Moreover, the background-subtracted mass spectrum corresponding to the *m/z*-130-ion peak confirmed that of the eight deuterium atoms present in the precursor *m*-cresol-d_8, six of them are incorporated to 2-methyl-1,4-hydroquinone-d_6 (Fig. 7a insets).

Evidently, *m*-cresol undergoes hydroxylation to 2-methyl-1,4-hydroquinone first (presumably in the secretory cells, by a NADPH and oxygen mediated biosynthetic process; Artigot et al. 2009), which then undergoes oxidation to 2-methyl-1,4-benzoquinone in the reaction chamber (Fig. 8). In an analogous manner, 1,4-hydroquinone is oxidized to 1,4-benzoquinone in the reaction chamber. When 1,4-hydroquinone-d_6 (Fig. 1f) was administered to *B. elongatulus* and the defensive gland extract was obtained after a few days and analyzed by GC-MS, the chromatographic peak for 1,4-benzoquinone was accompanied by another peak for 1,4-benzoquinone-d_4 (Fig. 9b). The presence of 1,4-benzoquinone-d_4 was confirmed by extracting a mass chromatogram for the *m/z* 112 ion (Supplementary Figure S4c). Moreover, the background-subtracted mass spectrum corresponding to the *m/z* 112 ion confirmed that of the six deuterium atoms present in the precursor, four of them have been incorporated to 1,4-benzoquinone-d_4 (Supplementary Figure S4a).

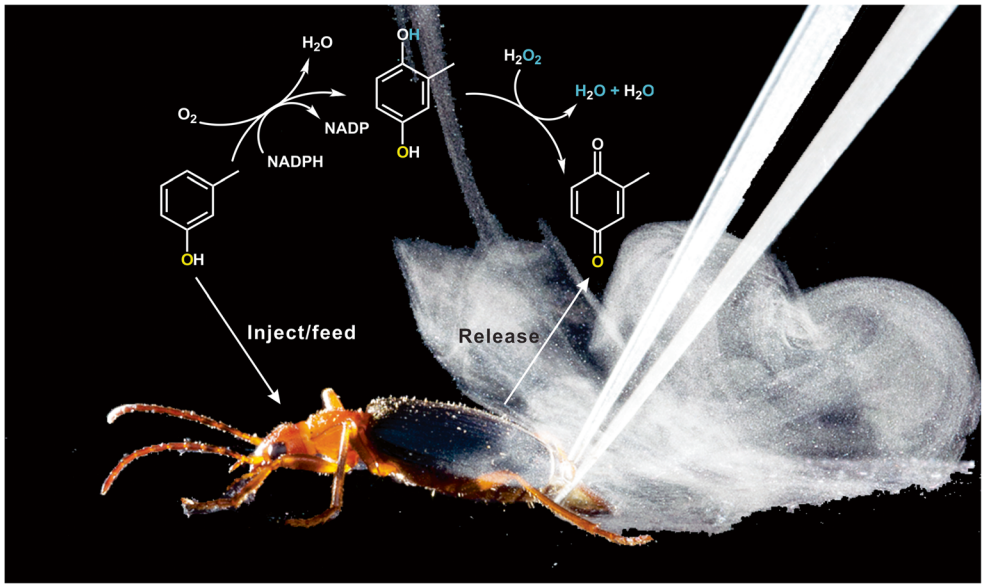

Fig. 10 Biosynthesis of 2-methyl-1,4-benzoquinone

Discussion

In 2000, Deml and Huth (2000) speculated that the biosynthetic processes of all benzoquinones originate from one core precursor. According to their proposal, 1,4-hydroquinone is the central molecule that gets metabolized and converted to all benzoquinones (Shear 2015). However, we predicted that 2-methyl-1,4-benzoquinone in bombardier beetles might not originate 1,4-benzoquinone. To test our hypothesis, we injected a solution of *m*-cresol-d_8 to several beetles. Subsequent chemical analysis showed that six deuterium atoms from *m*-cresol-d_8 were incorporated into 2-methyl-1,4-benzoquinone. Furthermore, our mass chromatographic analysis of data acquired from the defensive secretion of 1,4-hydroquinone-d_6-injected bombardier beetles revealed the presence of only one deuterium-labeled compound. In other words, the deuterium atoms from 1,4-hydroquinone-d_6 were specifically incorporated only into 1,4-benzoquinone and not into 2-methyl-1,4-benzoquinone or any other quinone present in the secretion. Since no other benzoquinone acquired the deuterium-labeling, we can conclude that each benzoquinone originates only from a specific hydroquinone precursor and not by an alkylating mechanism using 1,4-hydroquinone as the core precursor as suggested by Deml and Huth (2000). In addition, we confirm that bombardier beetles have the ability to convert *m*-cresol to 2-methyl-1,4-hydroquinone, which is then oxidized to 2-methyl-1,4-benzoquinone in their defensive spray (Fig. 10).

It is known that *m*-cresol is a cytotoxic compound that interacts with membrane lipids at the cellular level and causes membrane leakage (Paiva et al. 2016). However, *B. elongatulus* appears to be resistant to the ill effects of *m*-cresol. Whether the labeled *m*-cresol was provided in their food or via direct injection into the hemocoel, the beetles were able to convert it into hydroquinones stored in the reservoir. It is likely that the *m*-cresol added to their food passed from the digestive system through the midgut wall into the hemocoel and was transported through the hemolymph to the secretory cells of the reservoir. McManus et al. (2018) showed that the secretory cells and Malpighian tubules are always intertwined in *B. elongatulus* (see Fig. 2 in McManus et al. (2018)). This physical connection further suggests that the digestive system may also be somehow involved in the production of *Brachinus* defensive chemicals. Whatever the phenol transport pathway may be, the speed at which the beetles can incorporate *m*-cresol into benzoquinone—in as little as 24 h—is remarkable and attests to the importance of defensive chemistry for the survival of these beetles.

Funding information The authors received financial support from the National Science Foundation (NSF) grants 1556898 (ABA), 1556813 (WM), and 1556957 (KW).

Compliance with ethical standards

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

Statement on the welfare of animals All applicable international and national guidelines for the care and use of animals were followed.

References

Abraham I, Joshi R, Pardasani P, Pardasani RT (2011) Recent advances in 1,4-benzoquinone chemistry. J Braz Chem Soc 22:385–421

Aneshansley DJ, Eisner T, Widom JM, Widom B (1969) Biochemistry at 100°C: explosive secretory discharge of bombardier beetles (*Brachinus*). Science 165:61–63

Arndt EM, Moore W, Lee W-K, Ortiz C (2015) Mechanistic origins of bombardier beetle (Brachinini) explosion-induced defensive spray pulsation. Science 348:563–567

Arndt E, Beutel RG, Will KW (2017) Carabidae Latreille, 1802. In R. G. Beutel & R. A. B. Leschen (Eds.), Handbook of zoology, Vol. IV: Arthropoda: Insecta (series ed. by N.P. Kristensen and R.G. Beutel). Part 38. Coleoptera, Vol. 1: Morphology and systematics (Archostemata, Adephaga, Myxophaga, Polyphaga (partim). 2nd ed. New York: Walter De Gruyter, Berlin. pp 119-146

Artigot MP, Loiseau N, Laffitte J, Mas-Reguieg L, Tadrist S, Oswal IP, Puel O (2009) Molecular cloning and functional characterization of two CYP619 cytochrome P450s involved in biosynthesis of patulin in *Aspergillus clavatus*. Microbiology 155:1738–1747

Balestrazzi E, Dazzini-Valcurone ML, Bernardi MD, Vidari G, Vita-Finzi P, Mellerio G (1985) Morphological and chemical studies on the pygidial defence glands of some Carabidae (Coleoptera). Naturwissenschaften 72:482–484

Beynon JH, Williams AE (1960) Mass spectra of various quinones and polycyclic ketones. Appl Spectrosc 14:156–160

Blum M (1981) Chemical defenses of arthropods. Academic Press, Waltham

Blum MS (1987) Biosynthesis of arthropod exocrine compounds. Annu Rev Entomol 32:381–413

Bodner M, Vagalinski B, Makarov SE, Antić DŽ, Vujisić LV, Leis HJ, Raspotnig G (2016) "Quinone millipedes" reconsidered: evidence for a mosaic-like taxonomic distribution of phenol-based secretions across the Julidae. J Chem Ecol 42:249–258

Bradshaw JWS (1985) Insect natural products — compounds derived from acetate, Shikimate and amino acids. In: Kerkut GA, Gilbert LI (eds) Comprehensive insect physiology biochemistry and pharmacology, Pharmacology, vol 11. Elsevier, Amsterdam, pp 655–703

Buser H-R, Arn GP, Rauscher S (1983) Determination of double bond position in mono-unsaturated acetates by mass spectrometry of dimethyl disulfide adduct. Anal Chem 55:818–822

Chadha MS, Eisner T, Meinwald J (1961) Defence mechanisms of arthropods—IV: para-benzoquinones in the secretion of *Eleodes longicollis* Lec. (Coleoptera: Tenebrionidae). J Insect Physiol 7: 46–48

Dazzini-Valcurone M, Pavan M (1980) Glandole pigidiali e secrezioni difensive dei Carabidae (Insecta Coleoptera). Publicazioni dell'Ist Entomol Univ di Pavia 12:36

Dean J, Aneshansley DJ, Edgerton HE, Eisner T (1990) Defensive spray of the bombardier beetle: a biological pulse jet. Science 248:1219–1221

Deml R, Huth A (2000) Benzoquinones and hydroquinones in defensive secretions of tropical millipedes. Naturwissenschaften 87:80–82

Dettner K (1985) Ecological and phylogenetic significance of defensive compounds from pygidial glands of Hydradephaga Coleoptera. Proceedings of the Academy of Natural Sciences. 1st International Conference on Classification, Phylogeny, and Natural History of Hydradephaga, Held at the 1982 Joint Meeting of the Entomological Societies of America, Canada, and Ontario 137: 156–171

Dettner K (1991) Solvent-dependent variability of effectiveness of quinone-defensive systems of Oxytelinae beetles (Coleoptera: Staphylinidae). Entomologia Generalis 15:275–292

Dettner K (2010) Chemical defense and toxins of lower terrestrial and freshwater animals. In: Mori K (ed) Comprehensive natural products II: chemistry and biology, chemical ecology, vol 4. Elsevier, Amsterdam, pp 387–410

Di Giulio A, Muzzi M, Romani R (2015) Functional anatomy of the explosive defensive system of bombardier beetles (Coleoptera, Carabidae, Brachininae). Arthropod Struct Dev 44:468–490

Dufey SS (1974) The biosynthesis of defensive chemicals by millipedes: parallelism with plant biosynthetic pathways. Ph.D. thesis, The University of British Columbia

Eisner T (1958) The protective role of the spray mechanism of the bombardier beetle, *Brachynus ballistarius* Lec. J Insect Physiol 2:215–220

Eisner T, Aneshansley DJ (1982) Spray aiming in bombardier beetles: jet deflection by Coanda effect. Science 215:83–85

Eisner T, Jones TH, Aneshansley DJ, Tschinkel WR, Silberglied RE, Meinwald J (1977) Chemistry of defensive secretions of bombardier beetles (Brachinini, Metriini, Ozaenini, Paussini). J Insect Physiol 23:1383–1386

Eisner T, Rossini C, Eisner M (2000) Chemical defense of an earwig (*Doru taeniatum*). Chemoecology 10:81–87

Eisner T, Rossini C, González A, Eisner M (2004) Chemical defense of an opilionid (*Acanthopachylus aculeatus*). J Exp Biol 207:1313–1321

Fieser LF, Ardao MI (1955) Investigation of the chemical nature of gonyleptidine. J Amer Chem Soc 78:774–781

Francis GW, Veland K (1987) Alkylthiolation for the determination of double-bond positions in linear alkenes. J Chromatogr A 219:379–384

Gasch T, Schott M, Wehrenfennig C, Düring R-A, Vilcinskas A (2013) Multifunctional weaponry: the chemical defenses of earwigs. J Insect Physiol 59:1186–1193

Gnaspini P, Cavalheiro AJ (1998) Chemical and behavioral defenses of a neotropical cavernicolous harvestman: *Goniosoma spelaeum* (Opiliones, Laniatores, Gonyleptidae). J Arachnology 26:81–90

Happ GM (1968) Quinone and hydrocarbon production in the defensive glands of *Eleodes longicollis* and *Tribolium castaneum* (Coleoptera, Tenebrioidae). J Insect Physiol 14:1821–1837

Maddison DR, Moore W, Baker MD, Ellis TM, Ober KA, Cannone JJ, Gutell RR (2009) Monophyly of terrestrial adephagan beetles as indicated by three nuclear genes (Coleoptera: Carabidae and Trachypachidae). Zoological Scripta 38:43–62

McManus R, Ravenscraft A, Moore W (2018) Bacterial associates of a gregarious riparian beetle with explosive defensive chemistry. Front Microbiol 9:2361. https://doi.org/10.3389/fmicb.2018.02361

Meinwald J, Koch KF, Rogers JE Jr, Eisner T (1966) Biosynthesis of arthropod secretions. III. Synthesis of simple p-benzoquinones in a beetle (*Eleodes longicollis*). J Amer Chem Soc 88:1590–1592

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

Morgan D (2010) Biosynthesis in insects, Advances edn. Royal Society of Chemistry

Nojima S, Schal C, Webster FX, Santangelo RG, Roelofs WL (2005) Identification of the sex pheromone of the German cockroach, *Blattella germanica*. Science 307:1104–1106

Ober KA, Maddison DR (2008) Phylogenetic relationships of tribes within Harpalinae (Coleoptera: Carabidae) as inferred from 28S ribosomal DNA and the wingless gene. J Insect Sci 8:1–32

Olagbemiro TO, Lajide L, Sani KM, Staddon BW (1988) 2-Hydroxy-5-methyl-l,4-benzoquinone from the salivary gland of the soldier termites *Odontotermes magdalenae*. Experientia 44:1022–1024

Paiva TO, Bastos AEP, Marqu s JT, Viana AS, Lima PA, de Almeida RFM (2016) *m*-Cresol affects the lipid bilayer in membrane models and living neurons. RSC Adv 6:105699–105712

Raspotnig G, Schaider M, Föttinger P, Leutgeb V, Komposch C (2015) Benzoquinones from scent glands of phalangiid harvestmen (Arachnida, Opiliones, Eupnoi): a lesson from *Rilaena triangularis*. Chemoecology 25:63–72

Rocha DFO, Wouters FC, Zampieri DS, Brocksom TJ, Machado G, Marsaioli AJ (2013) Harvestman phenols and benzoquinones: characterisation and biosynthetic pathway. Molecules 18:11429–11451

Roth LM, Stay B (1958) The occurrence of *para*-quinones in some arthropods, with emphasis on the quinone-secreting tracheal glands of *Diploptera punctata* (Blattaria). J Insect Physiol 1:305–310

Schildknecht H (1957) Zur Chemie des Bombardierkäfers. Angew Chem Ger Ed 69:62–62

Schildknecht H, Holoubek K (1961) Die Bombardierk fer und ihre Explosionschemie V. Mitteilung über Insekten-Abwehrstoffe. Angew Chem Ger Ed 73:1–7

Schildknecht H, Koob K (1969) Zur Explosionschemie der Bombardierkäfer. Naturwissenschaften 56:328–328

Schildknecht H, Maschwitz E, Maschwitz U (1968) XXXIV. Mitteilung über Arthropodenabwehrstoffe. Die Explosionschemie der Bombardierkäfer (Coleoptera, Carabidae) III. Mitt.: Isolierung und Charakterisierung der Explosionskatalysatoren. Z Naturforsch B 23b:1213–1218

Schildknecht H, Maschwitz E, Maschwitz U (1970) Die Explosionschemie der Bombardierkäfer: Struktur und Eigenschaften der Brennkammerenzyme. J Insect Physiol 16:749–789

Schnepf E, Wenneis W, Schildknecht H (1969) Über Arthropoden-Abwehrstoffe XLI. Zur Explosionschemie der Bombardierkäfer (Coleoptera, Carabidae). Z Zellforsch 96:582–599

Sekulić TL, Vujisić LV, Ćurčić BPM, Mandić BM, Antić DŽ, Trifunović SS, Gođevac DM, Vajs VE, Tomić VT, Makarov SE (2014) Quinones and non-quinones from the defensive secretion of *Unciger transsilvanicus* (Verhoeff, 1899). (Diplopoda, Julida, Julidae), from Serbia. Arch Biol Sci Belgrade 66:385–390

Shear WA (2015) The chemical defenses of millipedes (Diplopoda): biochemistry, physiology and ecology. Biochem Syst Ecol 61:78–117

Thomson RH (1997) Naturally occurring quinones IV, recent advances. Chapman & Hall, London

Vuts J, Imrei Z, Birkett MA, Pickett JA, Woodcock CM, Tóth M (2014) Semiochemistry of the Scarabaeoidea. J Chem Ecol 40:190–210

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