1 **Benzoquinones in the defensive secretion of a bug (*Pamillia behrensii*): a common chemical**

2 **trait retrieved in the Heteroptera**

3 Julian M. Wagner[#], Thomas H. Naragon[#] and Adrian Brückner*

4 Division of Biology and Biological Engineering, California Institute of Technology, 1200 East

5 California Boulevard, Pasadena, CA 91125, United States of America

6 [#] both authors contributed equally

7 * corresponding author: adrian.brueckner@gmail.com

8 **Abstract**

9 Benzoquinones are a phylogenetically widespread compound class within arthropods,

10 appearing in harvestman, millipedes and insects. Whereas the function of benzoquinones as

11 defensive compounds against potential predators and microbes has been well established, the full

12 extent of benzoquinone usage across arthropods, and especially within Insecta, has yet to be

13 established. Adding to the growing list of unique evolutionary origins of benzoquinone

14 employment, we describe in this paper the metathoracic scent gland secretion of the mirid bug

15 *Pamillia behrensii*, which is composed of heptan-2-one, 2-heptyl acetate, 2,3-dimethyl-1-4-

16 benzoquinone, 2,3-dimethyl-1-4-hydroquinone as well as one unknown compound. Similarly, to

17 many other arthropods that use benzoquinones, *Pamillia* releases the contents of its gland as a

18 defensive mechanism in response to harassment by other arthropod predators. Morphological

19 investigation of the gland showed that the benzoquinone-producing gland complex of *P. behrensii*

20 follows a similar blueprint to metathoracic scent glands described in other Heteropterans. Overall,

21 our data further underpins the widespread convergent evolution and use of benzoquinones for

22 defense across the Arthropoda, now including the order Hemiptera.

1

23 **Keywords**

24   Quinones, chemical defense, exocrine glands, convergence, evolutionary biology

25 **Introduction**

26   Chemical convergence is the evolution and widespread use of certain compound classes by

27 different taxa for similar purposes (Beran et al. 2019; Brückner and Parker 2020). Evolutionary

28 patterns of this nature suggest potential genetic biases at the biosynthetic level or the efficiency of

29 certain compound classes at eliciting a given response in other organisms within certain ecological

30 settings as drivers of selection (Brückner and Parker 2020; Chevrette et al. 2020). '

31   One striking example of chemical convergence is the frequent evolution of aromatic

32 benzoquinones (BQs) as defensive compounds in arthropods (**Fig 1A**). The past 60 years of

33 research has uncovered a smorgasbord of quinone containing glands in insects, harvestment, and

34 millipedes (e.g., Blum 1981; Meinwald et al. 1966; Raspotnig et al. 2017; Roth and Stay 1958;

35 Shear 2015). In insects, BQs have a broad taxonomic distribution, spanning both hemi- and

36 holometabolous insects, with representatives in the earwigs (Dermaptera), lubber grasshoppers

37 (Orthoptera), cockroaches (Blattodea), caddisflies (Trichoptera) and most prominently beetles

38 (Coleoptera) (Blum 1981; Eisner et al. 2005; Francke and Dettner 2005).

39   Despite the similarity of the chemical phenotype, it is yet to be determined if the

40 biosynthetic pathways for the BQs are also convergent (Blum 1981; Morgan 2010). Some have

41 argued that BQs may be produced by enzymes related to tyrosine dependent cuticle tanning

42 (Duffey 1974; He et al. 2018; Roth and Stay 1958), while others have demonstrated the possibility

43 of *de novo* BQ synthesis from poly-β-carbonyls via head-to-tail condensation (Meinwald et al.

44 1966; Morgan 2010; Rocha et al. 2013). In contrast, the function of BQs in chemical defense

2

45   against predators and as antimicrobial agents is non-controversial and widely documented (e.g.,

46   Eisner et al. 2005; Gasch et al. 2013; Li et al. 2013; Ruther et al. 2001; Shear 2015).

47   Here we provide another example for the widespread evolution and chemical convergence

48   of benzoquinones, and report on the first evidence of this compound class in the Heteroptera

49   (Aldrich 1988). We chemically analyzed metathoracic scent gland (MTG) secretion of the mirid

50   bug *Pamillia behrensii,* Uhler (**Fig 2D**), investigated the internal and external morphology of the

51   gland via confocal microscopy and investigated the biological function of MTG compounds.

52   **Materials and Methods**

53   *Collection*

54   Individual *Pamillia behrensii* were collected in close proximity to nests of the velvety tree

55   ant *(Liometopum occidentale)* on October 23rd 2019 and November 17th 2020 at Chaney Canyon,

56   Altadena, CA (34°13'01.7"N 118°09'15.1"W). Insects were carefully transferred to the laboratory

57   in tubes lined with moist tissue paper and directly used for chemical analysis or imaging.

58   *Chemical analysis*

59   To identify compounds stored in the metathoracic scent gland (MTG) of *Pamillia*

60   *behrensii* we used whole body extraction in 50 µl hexane for 10 min, which is a well-established

61   method in Heteropterans (e.g., Leal et al. 1994; Zarbin et al. 2000). Crude hexane sample aliquots

62   (1 µl) were analyzed on a GCMS-QP2020 gas chromatography/mass-spectrometry system

63   (Shimadzu, Kyōto, Japan) equipped with a ZB-5MS fused silica capillary column (30 m x 0.25

64   mm ID, df= 0.25 µm) from Phenomenex (Torrance, CA, USA). Samples were injected using an

65   AOC-20i autosampler system from Shimadzu into a split/splitless-injector operating in splitless-

66   mode at a temperature of 310°C. Helium was used as the carrier-gas with a constant flow rate of

67   2.13 ml/min. The chromatographic conditions were as follows: The column temperature at the start

68   was 40°C with a 1-minute hold after which the temperature was initially increased 30°C/min to

69   250°C and further increased 50°C/min to a final temperature of 320°C and held for 5 minutes.

70   Electron impact ionization spectra were recorded at 70 eV ion source voltage, with a scan rate of

71   0.2 scans/sec from *m/z* 40 to 450. The ion source of the mass spectrometer and the transfer line

72   were kept at 230°C and 320°C, respectively.

73   We integrated chromatograms manually using LabSolutions Postrun Analysis (Shimadzu,

74   Kyōto, Japan), quantified the ion abundance and calculated the relative composition of individual

75   compounds compared to the total ion abundance. Compounds were identified based on the *m/z*

76   fragmentation patterns and retention indices (RIs) calculated after Van den Dool and Kratz (1963)

77   using a standard alkane mixture. Synthetic heptan-2-one was purchased from Sigma-Aldrich (St.

78   Louis, MO, USA) and natural 2-ethyl-1-4-benzoquinone and 2-ethyl-1-4-hydroquinone from the

79   secretion of *Tribolium castaneum* (Li et al. 2013) were used as reference compounds. 2-Heptyl

80   acetate was synthesized by mixing acetic acid with an excess of 2-heptanol and H_2SO_4 (all Sigma-

81   Aldrich) as catalyst and subsequently heating the mixture for 48 h at 45 °C. The crude ester was

82   extracted with hexane and the organic layer was washed with water, saturated Na_2CO_3 solution

83   (2x) and saturated NaCl solution and finally dried over anhydrous Na_2SO_4.

84   *Histochemistry and imaging*

85   Adult bugs were immersed in PBS and legs were removed with forceps. The dorsal parts

86   of the thorax were removed by cutting around the abdominal margin with dissection scissors and

87   the ventral thorax was fixed in 4% paraformaldehyde (25 minutes, room temperature), washed in

88   PBS+0.02% Triton X100, and subsequently stained with Alexa-647-Phalloidin (Thermo Fisher,

89   Waltham, MA, USA) to label muscles and Alexa-546-WGA (Thermo Fisher, Waltham, MA,

4

90   USA) to stain membranes. Metathoracic scent glands from both sides were imaged as whole

91   mounts of ventral thoraxes in ProLong Gold Antifade Mountant (Thermo Fisher, Waltham, MA,

92   USA), using a Zeiss LSM 880 (Carl Zeiss, Jena, Germany) with airyscan.

93   *Behavioral assay*

94   To confirm that P. behrensii deploys gland contents in response to threat we performed a

95   behavioral trial in tandem with solid phase microextraction (SPME). A 4-cm circular behavioral

96   arena was constructed from 1/8th inch infrared transmitting acrylic (Plexiglass IR acrylic 3143)

97   with a port on the side for a 65 µm polydimethylsiloxane/divinylbenzene fiber from Supelco

98   (Sigma-Aldrich; St. Louis, MO) to sample released volatiles. For compound desorption the fiber

99   was placed in the injector port for 1 min operating at 230°C and the GC/MS run was carried out

100   as outlined below. After each trial the fiber was baked for 30 min at 230°C. SPME-GC/MS runs

101   were used to identify the fraction of volatile metathoracic scent gland (MTG) compounds (see e.g.

102   Krajicek et al. 2016).

103   Behavior was recorded for thirty minutes at 25 fps with a machine vision camera (Flir

104   BFS-U3-16S2M-CS). Bugs were anesthetized on ice and placed in an arena either alone, with five

105   *L. occidentale*, or with five *L. occidentale* with superglued mandibles to reduce ant aggressiveness.

106   To assess behavior, bugs were labeled with DeepLabCut (DLC) to quantify movement over time

107   (Mathis et al. 2018). Median filtered, labeled bug locations were used to sum to total distance

108   traversed during the trial. SPME-GC/MS runs were used to identify the fraction of volatile

109   metathoracic scent gland (MTG) compounds (see e.g. Krajicek et al. 2016). A linear regression

110   was performed on 10,000 bootstrap samples of the data in python to calculate confidence intervals

111   for the regression slope. A Jupyter notebook is available which reproduces the analysis at

112   https://github.com/julianmwagner/bq_bug.

5

Results and Discussion

In total, we found five compounds in the MTG extracts of *Pamillia behrensii* of which we were able to identify four (**Fig 2A; Tab 1**). Compound I elicited a prominent fragment ion at $m/z=$ 58 arising from McLafferty rearrangement, which is characteristic for saturated methyl-ketones, together with a molecular ion at $m/z=$ 114 we identified I as heptan-2-one and eventually confirmed our identification with a synthetic standard.

Compound II showed a molecular ion at $m/z=$ 158 and a base ion at $m/z=$ 43 indicating an acetate ester [additional R'COOH$_2$ at $m/z=$ 61; Urbanová et al. (2012)] with a heptanol alcohol moiety (ion at m/z= 115). Both diagnostic ions at $m/z=$87 and the pair at $m/z=$69/70 indicate a possible heptan-2-ol moiety (**Tab 1**). Hence, we tentatively identified II as 2-heptyl acetate (1-methylhexyl acetate) and eventually confirmed the compound's identity by synthesis.

Compound III showed molecular ions at $m/z=$ 136 as well as diagnostic ions at $m/z=$ 108, $m/z=$ 82, $m/z=$ 79 and $m/z=$ 54, indicating either a 2,3-dimethyl- or 2-ethyl- substituted 1-4-benzoquinone. An ion at $m/z=$ 107 (i.e. 5-methyl-4-methylidenecyclopentenone ion) and III's retention index (**Tab 1**) were, however, more indicative of 2,3-dimethyl-1-4-benzoquinone. Compound V showed a molecular ions at $m/z=$ 138 (also the base ion) and diagnostic ions at $m/z=$ 137 $m/z=$ 95, as well as $m/z=$ 91 which together with V's retention index (**Tab 1**), were all indicative of 2,3-dimethyl-1-4-hydroquinone. We used the secretion from defensive stink gland of *Tribolium castaneum* (Li et al. 2013) which contains 2-ethyl-1,4-benzoquinone and 2-ethyl-1,4-hydroquinone as a natural standard and found no correspondence to III and V. Hence, we eventually identified III as 2,3-dimethyl-1,4-benzoquinone and V as 2,3-dimethyl-1,4-hydroquinone.

6

135   For compound IV we did not find any close hit in any compound library used (NIST14,

136   Wiley-NIST 2009, FCSN2) and the identity of this compound remains to be elucidated (see **Tab**

137   **1** for *m/z* fragmentation). The MTG extracts of *P. behrensii* (**Fig 2A**), overall showed a very

138   consistent chemical composition, with heptan-2-one always appearing in the greatest abundance,

139   2,3-dimethyl-1,4-benzoquinone at an intermediate abundance and 2-heptyl acetate, unknown IV

140   as well 2,3-dimethyl-1-4-hydroquinone being minor compounds (**Fig 2B; Tab 1**).

141   Based on synthetic heptan-2-one and 2-methyl-1,4-benzoquinone, as external standards,

142   we estimated 14.1±5.8 µg of heptan-2-one and 4.9±2.2 µg of 2,3-dimethyl-1,4-benzoquinone per

143   individual, respectively. Based on the composition and amounts, we interpreted heptan-2-one as a

144   solvent for the more noxious benzoquinone and hydroquinone, while the ester 2-heptyl acetate

145   may be an additional repellent or act as surfactant as previously described for glandular secretions

146   of rove beetles (Dettner 1984; Steidle and Dettner 1993).

147   Detecting 2,3-dimethyl-1,4-benzoquinone in the MTG extracts of a heteropteran (**Fig 2A**),

148   adds another order of arthropods to the list of BQ-producers and underpins the remarkable pattern

149   of chemical convergent evolution of BQs. Even though other aromatics compounds (e.g. vanillin,

150   2-phenylethanol, benzyl alcohol, or p-hydroxbenzaldehyde) have been described in other bugs,

151   benzoquinones add another compound class to the chemical skillset of the Heteroptera. Focusing

152   on aliphatic compounds, MTG secretions of bugs are typically composed of n-alkanes, alkenals,

153   alkenyl alkanoates and occasionally monoterpenoids. While heptan-2-one appears to be a rather

154   unusual compound for bugs as well, methyl ketones are common in arthropods. For instance, in

155   ants, other hymenopterans, trichopterans as well as cyphophthalmid and dyspnoan harvestman

156   they serve as alarm and sex pheromones or as defensive compounds (Blum 1981; Cheng et al.

157   2017; Löfstedt et al. 2008; Raspotnig et al. 2005; Schaider et al. 2018).

158   2,3-Dimethyl-1-4-hydroquinone in the MTG secretions of *P. behrensii* (**Fig 2A**) can be

159   interpreted as a precursor molecule that is oxidized to the final benzoquinone *via* a laccase

160   enzymes, as a recent transcriptomic study in termite soldiers suggested (He et al. 2018). Whether

161   the hydroquinone is derived from tyrosine or was produced utilizing a polyketide-like mechanism

162   (Brückner and Parker 2020) is, however, unknown.

163   As is typical for chemical release from the metathoracic scent gland of other bugs, we

164   found that the yellow, quinone secretion is also expelled from this gland complex as small droplets

165   (**Fig 2C**). The MTG was located between thorax segment II and III (**supplement Fig S1A**) and

166   generally showed an outer morphology comparable to other Heteropteran glands (Aldrich 1988;

167   Gonzaga-Segura et al. 2013; Hepburn and Yonke 1971): two adjacent cuticular folds form

168   flaps/lips on the anterior and posterior edges of the gland opening (**Fig 2C**). The edges of the

169   cuticular folds constitute the ostiolar peritreme, which likely aids in carrying the glandular

170   secretions away from the opening and fosters increased compound dispersion (**Fig 2C**).

171   Additionally, the roughly sculpted cuticle around the gland opening serves as an evaporation

172   surface (**Fig 2C**), increasing the volatility of expelled MTG compounds (Aldrich 1988; Hepburn

173   and Yonke 1971).

174   The internal part of the MTG complex (**Fig S1B and C**) consists of a lateral extension

175   which connects the gland opening to the median reservoir where the secretions produced by

176   primary gland are stored (Aldrich 1988; Hepburn and Yonke 1971). Despite the capability of the

177   MTG of *P. behrensii* to synthesize quite unusual Heteropteran compounds, the general outer and

178   inner morphology of the MTG complex appears to be similar to that found in other MTG

179   containing species and we did not find any specialized adaptions to house these compounds.

8

180   To confirm *P. behrensii* uses its MTG secretion (**Fig 2A**) as a defensive compound,

181   we performed behavioral experiments coupled with chemical headspace profiling with solid phase

182   microextraction. We expected the chemical secretion expelled from the gland should function as

183   an allomone in chemical defense, as quinones are known to be noxious and serve as predator

184   repellents in many other arthropods (e.g., Eisner et al. 2005; Gasch et al. 2013; Li et al. 2013;

185   Ruther et al. 2001). Like other chemicals, benzoquinones activate the transient receptor potential

186   ankyrin 1 (TRPA1), a nonselective cation channel, which has a conserved function as a noxious

187   chemical receptor in animals (Arenas et al. 2017; Ibarra and Blair 2013). TRPA1 might thus be a

188   major target of many convergently evolved defensive chemicals like benzoquinones and initiate

189   protective behavior after a chemical attack (Blair et al. 2016; Ibarra and Blair 2013).

190   We found direct evidence of active usage of the MTG gland for defense in our behavioral

191   recordings. In cases where ants successfully grabbed the bug, deployed gland content could be

192   seen smeared on the behavioral arena (**Fig 3A**). The compounds quickly evaporated off the arena

193   surface and were readily measurable *via* SPME-GCMS. We additionally profiled the correlation

194   of amount of benzoquinone deployed with bug movement in the behavioral arena (**Fig 3B**). We

195   found a positive correlation (95% confidence interval of slope: 0.154-1.75) of BQ amount with the

196   distance the bug traveled during the behavioral trial (a proxy bug agitation/escape response). This

197   behavioral readout supports the idea that *P. behrensii* deploys its gland to confuse or repel its

198   predators before fleeing. We thus demonstrated that *P. behrensii* uses the compounds stored in its

199   MTG as a repellent against predators, which again underpins the chemical convergence of

200   benzoquinones.

201   We found first evidence of a benzoquinone in the Heteroptera (Aldrich 1988; Morgan

202   2010). More specifically we detected 2,3-dimethyl-1-4-benzoquinone produced by and stored in

9

203 the metathoracic scent gland complex of the mirid bug *Pamillia behrensii* and showed that it uses

204 this BQ in combination with three other compounds for chemical defense against predators. Our

205 study therefore highlights the remarkable convergent evolution of benzoquinones as defensive

206 compounds across the Arthropoda, now including true bugs.

Acknowledgment

Ethics statement

211   There are no legal restrictions on working with bugs. Field collection permissions were

212 issues by California Department of Fish and Wildlife and the Angeles National Forest (US Forest

213 Service; USDA).

Authors contributions

215   JMW, THN and AB design research; JMW and THN collected specimens; THN performed

216 microscopy; JMW performed and analyzed behavioral assays; JMW collected chemical data; AB

217 analyzed chemical data and performed organic synthesis; AB supervised the project; AB wrote the

218 paper with input from THN and JMW. JMW and THN contributed equally. All authors gave final

219 approval for publication.

References

220 **References**

221 Aldrich J (1988) Chemical ecology of the Heteroptera Annual Review of Entomology 33:211-238

222 Arenas OM, Zaharieva EE, Para A, Vásquez-Doorman C, Petersen CP, Gallio M (2017) Activation

223   of planarian TRPA1 by reactive oxygen species reveals a conserved mechanism for animal

224   nociception Nature neuroscience 20:1686-1693

225 Beran F, Köllner TG, Gershenzon J, Tholl D (2019) Chemical convergence between plants and

226   insects: biosynthetic origins and functions of common secondary metabolites New

227   Phytologist

228 Bertoli A, Leonardi M, Krzyzanowska J, Oleszek W, Pistelli L (2011) Mentha longifolia in vitro

229   cultures as safe source of flavouring ingredients Acta Biochimica Polonica 58

230 Blair NT, Philipson BI, Richards PM, Doerner JF, Segura A, Silver WL, Clapham DE (2016)

231   Naturally produced defensive alkenal compounds activate TRPA1 Chemical senses

232   41:281-292

233 Blum MS (1981) Chemical Defenses of Arthropods Academic Press, Inc., New York

234 Brückner A, Parker J (2020) Molecular evolution of gland cell types and chemical interactions in

235   animals Journal of Experimental Biology 223

236 Cheng Y-n, Wen P, Dong S-h, Tan K, Nieh JC (2017) Poison and alarm: the Asian hornet Vespa

237   velutina uses sting venom volatiles as an alarm pheromone Journal of experimental biology

238   220:645-651

239 Chevrette MG et al. (2020) Evolutionary dynamics of natural product biosynthesis in bacteria Nat

240   Prod Rep 37:566-599

241 Dettner K (1984) Isopropylesters as wetting agents from the defensive secretion of the rove beetle

242   Coprophilus striatulus F.(Coleoptera, Staphylinidae) Insect biochemistry 14:383-390

243   Duffey SS (1974) The biosynthesis of defensive chemicals by millipedes: parrallelism with plant

244   biosynthetic pathways. University of British Columbia

245   Eisner T, Eisner M, Siegler M (2005) Secret Weapons. Secret Weapons. Harvard University Press,

246   Cambridge

247   Francke W, Dettner K (2005) Chemical signalling in beetles. In: The Chemistry of Pheromones

248   and Other Semiochemicals II. Springer, pp 85-166

249   Gasch T, Schott M, Wehrenfennig C, Düring R-A, Vilcinskas A (2013) Multifunctional weaponry:

250   the chemical defenses of earwigs Journal of insect physiology 59:1186-1193

251   Gonzaga-Segura J, Valdez-Carrasco J, Castrejon-Gomez VR (2013) The metathoracic scent gland

252   of the leaf-footed bug, Leptoglossus zonatus Journal of Insect Science 13:149

253   He S et al. (2018) Termite soldiers contribute to social immunity by synthesizing potent oral

254   secretions Insect molecular biology 27:564-576

255   Hepburn H, Yonke T (1971) The metathoracic scent glands of coreoid Heteroptera Journal of the

256   Kansas Entomological Society:187-210

257   Ibarra Y, Blair NT (2013) Benzoquinone reveals a cysteine-dependent desensitization mechanism

258   of TRPA1 Molecular pharmacology 83:1120-1132

259   Krajicek J et al. (2016) Comparative analysis of volatile defensive secretions of three species of

260   Pyrrhocoridae (Insecta: Heteroptera) by gas chromatography-mass spectrometric method

261   PloS one 11:e0168827

262   Leal WS, Panizzi AR, Niva CC (1994) Alarm pheromone system of leaf-footed bugLeptoglossus

263   zonatus (Heteroptera: Coreidae) Journal of Chemical Ecology 20:1209-1216

12

264   Li J, Lehmann S, Weißbecker B, Naharros IO, Schütz S, Joop G, Wimmer EA (2013) Odoriferous

265   defensive stink gland transcriptome to identify novel genes necessary for quinone synthesis

266   in the red flour beetle, Tribolium castaneum PLoS genetics 9:e1003596

267   Löfstedt C, Bergmann J, Francke W, Jirle E, Hansson BS, Ivanov VD (2008) Identification of a

268   sex pheromone produced by sternal glands in females of the caddisfly Molanna angustata

269   Curtis Journal of chemical Ecology 34:220

270   Mathis A, Mamidanna P, Cury KM, Abe T, Murthy VN, Mathis MW, Bethge M (2018)

271   DeepLabCut: markerless pose estimation of user-defined body parts with deep learning

272   Nature neuroscience 21:1281-1289

273   Meinwald J, Koch KF, Rogers Jr JE, Eisner T (1966) Biosynthesis of arthropod secretions. III.

274   Synthesis of simple p-benzoquinones in a beetle (*Eleodes longicollis*) Journal of the

275   American Chemical Society 88:1590-1592

276   Misof B et al. (2014) Phylogenomics resolves the timing and pattern of insect evolution Science

277   346:763-767

278   Morgan ED (2010) Biosynthesis in Insects. RSC Publishing, Camebridge

279   Raspotnig G, Fauler G, Leis M, Leis HJ (2005) Chemical profiles of scent gland secretions in the

280   cyphophthalmid opilionid harvestmen, *Siro duricorius* and *S. exilis* Journal of Chemical

281   Ecology 31:1353-1368 doi:10.1007/s10886-005-5291-4

282   Raspotnig G, Schaider M, Föttinger P, Schönhofer A (2017) A model for phylogenetic

283   chemosystematics: evolutionary history of quinones in the scent gland secretions of

284   harvestmen Frontiers in ecology and evolution 5:139

285 Rocha D, Wouters F, Zampieri D, Brocksom T, Machado G, Marsaioli A (2013) Harvestman

286   phenols and benzoquinones: characterisation and biosynthetic pathway Molecules

287   18:11429-11451

288 Roth LM, Stay B (1958) The occurrence of para-quinones in some arthropods, with emphasis on

289   the quinone-secreting tracheal glands of *Diploptera punctata* (Blattaria) Journal of Insect

290   Physiology 1:305-318

291 Ruther J, Podsiadlowski L, Hilker M (2001) Quinones in cockchafers: additional function of a sex

292   attractant as an antimicrobial agent Chemoecology 11:225-229

293 Schaider M, Novak T, Komposch C, Leis H-J, Raspotnig G (2018) Methyl-ketones in the scent

294   glands of Opiliones: a chemical trait of Cyphophthalmi retrieved in the dyspnoan

295   Nemastoma triste Chemoecology 28:61-67

296 Shear WA (2015) The chemical defenses of millipedes (diplopoda): Biochemistry, physiology and

297   ecology Biochemical Systematics and Ecology 61:78-117 doi:10.1016/j.bse.2015.04.033

298 Steidle JLM, Dettner K (1993) Chemistry and morphology of the tergal gland of freeliving adult

299   Aleocharinae (Coleoptera: Staphylinidae) and its phylogenetic significance Systematic

300   Entomology 18:149-168

301 Stork NE (2018) How many species of insects and other terrestrial arthropods are there on Earth?

302   Annual review of entomology 63:31-45

303 Urbanová K, Vrkoslav V, Valterová I, Háková M, Cvacka J (2012) Structural characterization of

304   wax esters by electron ionization mass spectrometry Journal of lipid research 53:204-213

305 Zarbin PH, Borges M, Santos AAd, de Oliveira AR, Simonelli F, Marques FdA (2000) Alarm

306   pheromone system of stink bug Piezodorus guildinii (Heteroptera: Pentatomidae) Journal

307   of the Brazilian Chemical Society 11:424-428

308

309   **Table 1** Gas chromatographic and mass spectrometric data of the metathoracic scent gland secretion released by the mirid bug *Pamillia*

310   *behrensii*. Molecular ions (M+) are marked in bold. Retention indices (RI) were calculated according to van den Dool and Kratz (1963).

RI	RI literature	Identified as	Mass spectrometric fragmentation *m/z* (relative intensity)	Secretion profile [%]
890	892[a]	heptan-2-one	114 (M^+, 4), 99 (3), 85 (3), 71 (15), 58 (55), 43 (100)	64.1±5.2
1041	1043[b]	2-heptyl acetate	158 (M^+, 1), 115 (3), 102 (2), 98 (7), 87 (22), 70 (8), 69 (7), 61 (3), 56 (15), 43 (100)	6.5±6.5
1118	1119[c]	2,3-dimethyl-1-4-benzoquinone	136 (M^+, 85), 108 (71), 107 (59), 82 (59), 80 (31), 79 (76), 54 (100), 53 (44)	26.5±4.5
1223	-	unknown	138 (M^+, 52), 110 (14), 95 (16), 82 (29), 67 (40), 54 (100)	2.1±1.6
1432	1433[c]	2,3-dimethyl-1-4-hydroquinone	138 (M^+, 100), 137 (25), 123 (53), 95 (29), 91 (25)	0.8±0.7

311

312   [a]RI as reported by Schaider et al. (2018)

313   [b]RI as reported by Bertoli et al. (2011)

314   [c]RI as reported by Rocha et al. (2013)

15

315 **Figure 1 Convergent evolution of benzoquinones across the arthropods.** Conceptual

316 phylogenetic tree of arthropods - focusing on insects - depicting taxa producing benzoquinones

317 based on previous research (in red) and results presented in this study (in orange). The tree

318 topology is based on Misof et al. (2014) and the group size is proportional to the number of species

319 based on Stork (2018).

320 **Figure 2 Metathoracic scent gland (MTG) chemistry of *Pamillia behrensii*. A:** Example GC

321 traces of *P. behrensii* gland exudates. Compounds are labeled in the trace and mass spectrometric

322 data is detailed in Table 1. **B:** Matrix plot shows the composition of eleven individuals. The gland

323 compounds in the order of their retention indices are heptan-2-one, 2-heptyl acetate, 2,3-dimethyl-

324 1,4-benzoquinone, unknown IV and 2,3-dimethyl-1,4-hydroquinone. The legend indicates the

325 percentage composition for each compound per specimen. **C:** Outer morphology of MTG of *P.*

326 *behrensii.* The top image shows a droplet of the glandular secretion shortly after gland use. The

327 yellow color indicates the presence of benzoquinones. The bottom image details the surface

328 morphology around the gland opening. op= gland opening; evp= evaporation surface; per=

329 peritreme; can= canal from ostiole to coxal pit. **D:** Habitus image of *P. behrensii* with scale bar.

330 **Figure 3 Behavioral evidence active defense gland use against predators. A:** When placed in

331 a behavioral arena ants attack *Pamillia behrensii* (left panel of A). In response to aggression, *P.*

332 *behrensii* deploys defensive gland secretion, which can be seen by eye (A central panel, indicated

333 with arrow). The gland secretion volatilizes, diffusing to nearby attackers (A right panel); volatile

334 compound identity was confirmed *via* SPME-GCMS. **B:** Quantitative analysis of behavior

335 indicated a significant positive correlation of bug movement (a proxy for bug agitation/attempts to

336 flee predators) with benzoquinone quantity deployed. Grey intervals represent 95th, 75th and 10th

337 percentile for regression confidence intervals from 10,000 bootstrap replicates. Together, these

16

338   suggest that *P. behrensii* employs a joint strategy of gland deployment with an escape response to

339   evade aggressive predators.

340   **Supplementary Figure S1** Overview of the morphology of the *P. behrensii* gland. **A:** Location

341   of the metathoracic scent gland (MTG) opening located between thorax segment II and II. **B + C:**

342   Inner morphology of the MTG showing the median reservoir where the secretions produced by

343   primary gland are stored.

344

CHELICERATA
MYRIAPODA
PROTURA
COLLEMBOLA
DIPLURA
ARCHAEOGNATHA
ODONATA
EPHEMEROPTERA
ZORAPTERA
DERMAPTERA
PLECOPTERA
ORTHOPTERA
EMBIOPTERA
GRYLLOBLATTODEA
MANTOPHASMATODEA
PHASMATODEA
MANTODEA
BLATTODEA
THYSANOPTERA

HEMIPTERA

PSOCODEA

HYMENOPTERA

RAPHIDIOPTERA
MEGALOPTERA

NEUROPTERA

STREPSIPTERA

COLEOPTERA

TRICHOPTERA

LEPIDOPTERA

SIPHONAPTERA
MECOPTERA

DIPTERA

(A)

(B)

I - heptan-2-one
II - 2-heptyl acetate
III - 2,3-dime-1,4-BQ
IV - unknown
V - 22,3-dime-1,4-HQ

(C)

evp
op
can
per

(D)

(A) ant attack | gland used | volatiles diffuse

(B)

- bug alone
- bug + 5 ants glued
- bug + 5 ants

x-axis: log(cm traveled by bug during trial)
y-axis: log(BQ counts)