Chemical characterisation of potential pheromones from the shoulder gland of the Northern yellow-shouldered-bat, *Sturnira parvidens* (Phyllostomidae: Stenodermatinae)

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Bats of the genus *Sturnira* (Family Phyllostomidae) are characterised by shoulder glands that are more developed in reproductively mature adult males. The glands produce a waxy secretion that accumulates on the fur around the gland, dyeing the fur a dark colour and giving off a pungent odour. These shoulder glands are thought to play a role in their reproductive behaviour. Using gas chromatography–mass spectrometry, we analysed solvent extracts of fur surrounding the shoulder gland in the northern-shouldered bat,*Sturnira parvidens*to (i) characterise the chemical composition of shoulder gland secretions for the first time, and (ii) look for differences in chemical composition among and between adult males, sub-adult/juvenile males and adult females. Fur solvent extracts were analysed as liquids and also further extracted using headspace solid-phase microextraction to identify volatile components in the odour itself.Odour fingerprint analysis using non-metric multidimensional scaling (NMDS) plots and multivariate analysis revealed clear and significant differences (P<0.001) between adult males versus both juvenile males and adult females. The chemical components of the shoulder gland secretion included terpenes and phenolics, together with alcohols and esters, most likely derived from the frugivorous diet of the bat. Many of the compounds identified were found exclusively or in elevated quantities among adult (reproductive) males compared with adult females and non-reproductive (juvenile) males. This strongly suggests a specific role in male–female attraction although a function in male-male competition and/or species recognition is also possible.

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27 **Abstract**

28 Bats of the genus *Sturnira* (Family Phyllostomidae) are characterised by shoulder glands that are

29 more developed in reproductively mature adult males. The glands produce a waxy secretion that

30 accumulates on the fur around the gland, dyeing the fur a dark colour and giving off a pungent

31 odour. These shoulder glands are thought to play a role in their reproductive behaviour. Using

32 gas chromatography–mass spectrometry, we analysed solvent extracts of fur surrounding the

33 shoulder gland in the northern-shouldered bat, *Sturnira parvidens* to (i) characterise the chemical

34 composition of shoulder gland secretions for the first time, and (ii) look for differences in

35 chemical composition among and between adult males, sub-adult/juvenile males and adult

36 females. Fur solvent extracts were analysed as liquids and also further extracted using headspace

37 solid-phase microextraction to identify volatile components in the odour itself. Odour fingerprint

38 analysis using non-metric multidimensional scaling (NMDS) plots and multivariate analysis

39 revealed clear and significant differences (P<0.001) between adult males versus both juvenile

40 males and adult females. The chemical components of the shoulder gland secretion included

41 terpenes and phenolics, together with alcohols and esters, most likely derived from the

42 frugivorous diet of the bat. Many of the compounds identified were found exclusively or in

43 elevated quantities among adult (reproductive) males compared with adult females and non-

44 reproductive (juvenile) males. This strongly suggests a specific role in male–female attraction

45 although a function in male-male competition and/or species recognition is also possible.

46

47 **Introduction**

48 Pheromones are intra-specific semiochemicals (chemical signals) that modify the behaviour

49 and/or physiology in the recipient animal. They occur across the animal kingdom and are

50 particularly well characterised in insects. Among mammals, there are many examples of

51 pheromonal communication (see Wyatt, 2014 for recent review), but their chemical composition

52 and actions in mammals has been debated (Doty, 2010). There are two general categories of

53 semiochemicals within a species: pheromones, chemical signals which elicit a stereotypical

54 behavioural or physiological response in the recipient animal, while signature mixtures are odour

55 cues which are learned by the conspecific receivers and often have complex and variable

56 chemical profiles. These "individual mixtures" or "signature odours", which act in social

57 communication though learning may identify a specific individual or social group (Wyatt 2014,

58 2017; Dehnhard 2011).

59   Bats (order Chiroptera), are the second most speciose mammalian order (after Rodentia),

60 yet relatively little is known about the possible role of pheromonal communication in this group.

61 Bats exhibit a wide variety of social and mating systems, implying the need for individual

62 recognition within groups, and exchange of social and reproductive cues (including mate choice).

63 A nocturnal lifestyle and potentially limited visual cues, particularly in roosts, could exaggerate

64 the importance of auditory and olfactory cues. Chemical cues are known to be used by big brown

65 bats *Eptesicus fuscus* (Bloss et al., 2002), and implicated in Bechstein's bats, *Myotis bechsteinii*

66 (Safi & Kirth, 2003) and the fisherman bat, *Noctilio leporinus* (Brooke & Decker, 1996).

67   While the exquisitely complex auditory systems associated with echolocation in bats

68 have been studied for years, work on their olfactory systems is more limited. Both the primary

69 and secondary olfactory systems are involved in pheromonal chemoreception, with the latter

70   forming the vomeronasal organ (VNO). A comparative anatomical study by Wible and

71   Bhatnagar (1996) looked across more than a hundred genera of bats and found extensive

72   variation in the presence or absence of the VNO, implying convergent gains and losses of the

73   structure. More recently, genomic studies have investigated the genes coding for olfactory

74   receptor proteins. Young *et al.* (2010) reported that two bat species (the little brown bat, *Myotis*

75   *lucifugus*, and the flying fox, *Pteropus vampyrus*) completely lacked intact genes for functional

76   receptors (V1R) in the VNO and they identified only inactive pseudogenes. Furthermore,

77   inactivating mutations in the vomeronasal signal transduction gene Trpc2 were also

78   characterised, suggesting that any VNO or secondary olfactory epithelia (if present) would be

79   non-responsive to pheromonal signals. Yohe et al. (2017) used Trpc2 as a molecular marker for

80   examining evolutionary losses and gains of the VNO system in more than 100 species across 17

81   of the 21 extant bat families. They found that all bats examined exhibit degraded vomeronasal

82   systems, except for some members of Miniopteridae and Phyllostomidae. The *Sturnira lilium*

83   species complex, which includes our study species *Sturnira parvidens*, does have a VNO and

84   does not have a pseudogenisation in Trpc2 exon 2, which would prevent signal transduction

85   (Yohe et al. 2017), suggesting a functional VNO and a potential role for pheromonal signalling

86   in this group. Collectively, these anatomical and genetic studies indicate that some, but not all,

87   bat species have the anatomical and physiological substrates required in the accessory olfactory

88   system for potentially receiving and responding to pheromones. However, pheromones in

89   mammals are also detected by the main olfactory system (Wyatt 2014, 2017) so the lack of

90   functioning VNO receptors does not rule out the possibility of semiochemical signaling.

91   Evidence for olfaction acting as a sensory modality for individual discrimination in bats

92   has also been gathered from behavioural studies. Scent-choice experiments by Bouchard (2001)

93   demonstrated that two African molossid bats (*Mops condylurus* and *Chaerephon pumilus*) can

94   distinguish between the sexes and distinguish roost-mates from strangers using olfactory cues. A

95   number of species in the family Emballonuridae have sacs in their wing or tail membranes,

96   which may function in a scent-based identification social system. These sacs are typically larger

97   and better developed in adult males. In the best-studied example by Voigt and von Helversen

98   (1999), male *Saccopteryx bilineata* keep harems of females and use their paired wing sacs

99   (located near the forward edge of each wing) to store bodily fluids. These fluids are then sprayed

100   on females during hovering or used in characteristic "salting" behaviour. Females also possess

101   wing sacs but they are rudimentary and the same behaviours have not been observed (Voigt &

102   von Helversen, 1999).

103   Despite observations that imply important roles for olfaction in social behaviour of bats,

104   there are few papers that identify the chemical make-up of chiropteran pheromones. Bloss et al.

105   (2002) found that female big brown bats, *Eptesicus fuscus* (Family Vespertilionidae) use

106   chemical cues to distinguish among female conspecifics, and these authors potentially identified

107   14 compounds based on analysis of their retention index on the gas chromatograph. Similarly,

108   Caspers et al. (2008) and later Schneeberger et al. (2016) established that the odour

109   "fingerprints" presented to females by male *Saccopteryx bilineata* contained differences

110   associated with age, colony, and year of sample collection. Thus, these signals could provide

111   information to females for assessing potential male mates. However, Schneeberger et al. (2016)

112   did not report the actual chemical composition of the wing-sac liquids. Other species of bats have

113   different structures that seem to produce odoriferous secretions possibly used in social

114   communication. Many species of molossids as well as the phyllostomid *Trachops cirrhosis* have

115   a single gland in the ventral midline of the throat which is found either exclusively in males or is

116   best developed in adult males (Bowles et al., 1990; Scully et al., 2000; Tandler et al., 1999;

117   Phillips et al., 1987). Male *Noctilio leporhinus* (Family Noctioionidae) secrete a strongly

118   smelling sticky substance along their lateral fur (N. Simmons, personal observation, 2017). The

119   chemical composition of these secretions has never been investigated.

120   The shoulder glands of *Sturnira* species are well known but understudied. *Sturnira* are

121   widely distributed and abundant from north-western Mexico, through Central America and into

122   tropical and subtropical South America (to the north of Argentina and Uruguay) and the Lesser

123   Antilles (Gannon et al., 1989; Velazco & Patterson, 2013; 2014). The common name of this

124   genus (yellow-shouldered bats) refers to glandular scent organs on the shoulder that produce a

125   yellowish or reddish staining of the fur that surrounds them (Figure 1). These glands are most

126   highly developed in males, and development of these glands appears to be dependent on the

127   reproductive maturity of the individual (Scully et al. 2000). In mature males they are described

128   as emitting a "strong, sweetish, musky odour" (Gannon et al., 1989; Goodwin & Greenhall,

129   1961) that can be so strong it is detectable by humans several metres away (E Clare, unpublished

130   observation).

131   Histological examination of the shoulder glandular region of male *Sturnira* revealed

132   numerous hair follicles and associated sebaceous glands, with scattered small sudoriferous

133   glands in a deeper layer of adipose tissue. The same glandular elements were also present in

134   females, although the size of the glandular region varied between males and females. Females

135   lack both the strong odour and strongly coloured hairs stained from secretion (Scully et al.,

136   2000). The production of a strongly-smelling secretion in reproductively mature males implies a

137   potential pheromonal role for these odours in reproduction, although very little has been reported

138 about the social or mating system of these species, and no behavioural or chemical analysis has

139 been done.

140   Our goal was to chemically characterise, for the first time, the secretions from the

141 shoulder gland of the northern yellow-shouldered bat, *Sturnira parvidens*. Using a gas

142 chromatography–mass spectrometry (GC–MS) approach, we identify the constituents and

143 compare the odour profiles among adult males, juvenile (non-reproductive) males, and adult

144 females.

145

146 **Methods**

147 **Sample collection and storage**

148 We conducted field work and collected samples in 2015 and 2016 at sites in Orange Walk

149 District, Belize, including the Lamanai Archaeological Reserve and adjacent secondary forest

150 and gardens near the Lamanai Outpost Lodge (17.75117 N, -88.65446 W), and a forest fragment

151 at the Ka'kabish Archaeological Project (17.8147 N, 148 -88.73052 W) Our research was

152 conducted in accordance with accepted standards for humane capture and handling of bats

153 published by the American Society of Mammalogists (Sikes *et al.* 2016) and approved

154 Institutional Animal Care and Use Committee protocols (Brown University IACUC 1205016 and

155 1504000134). Locally, the fieldwork was carried out with Belize Forestry Department Scientific

156 Research and Collecting Permits CD/60/3/15 (20) and WL/1/1/16 (26) respectively. All samples

157 were collected during a 10-day field trip at the end of April and the start of May to control for

158 any potential seasonal variation. We captured bats using mist nets set along forest paths, and

159 each individual was briefly contained in a cloth "bat bag" for identification and processing. We

160 determined sex and then checked for age and reproductive maturity in males by examining the

161   genitalia and ossification of the wing joints, following methods described by Racey (1988) and

162   Anthony (1988): males were classified as reproductive based on the size and descent of testes

163   into the scrotal sac, and as an adult if wing joints were ossified. We removed a small sample of

164   fur of the same size (approximately 4 mm^2) from an equivalent area surrounding the shoulder

165   gland on all individuals, using sterile scissors. More specifically, fur was collected from a

166   discoloured patch where oil was being secreted in adult males (Figure 1), while for adult females

167   and juveniles we collected fur from the same physical location as the adult males (which is often

168   also slightly discoloured). After fur was collected, we released all bats at the site of their capture.

169   Each fur sample was stored in a 2 ml screw-topped Eppendorf tube containing 1 mL of glyceryl

170   trioctanoate (Sigma-Aldrich, Gillingham, Dorset, UK), a medium-chain triglyceride (MCT), at

171   ambient temperature for later chemical analysis (within 2 months). The MCT acts as a solvent,

172   stabilizing and dissolving the organic components of any secretions from the shoulder gland.

173

174   **Direct injection of MCT extracts**

175   MCT extracts were analysed by gas chromatography–mass spectrometry (GC–MS), using an

176   Agilent 7693 autoinjector and Agilent 6890N gas chromatograph with 5975 mass spectrometer

177   (Agilent, Santa Clara, CA). The GC column used was a 15 m × 0.25 mm HP-5MS UI column

178   (0.25 μm film thickness; Agilent) and injection was in split mode (10:1) at 250 °C, the injection

179   volume being 1 μL. The initial temperature of the GC oven was 100 °C (0 min), rising at 5 °C

180   per min to 320 °C. Helium was used as the carrier gas at a constant flow rate of 1.1 mL/min. A

181   series of n-alkanes (C_5–C_{30}) in diethyl ether were analysed, under the same conditions, to obtain

182   linear retention index (LRI) values for the components of the extract.

183   The mass spectrometer operated in electron impact mode with an electron energy of 70

184   eV, scanning from *m/z* 29 to *m/z* 800 at 1.9 scans/s. We identified compounds by first comparing

185   their mass spectra with those contained in the NIST/EPA/NIH Mass Spectral Database or in

186   previously published literature. Wherever possible identities of compounds were confirmed by

187   comparison of linear retention index (LRI) values, with either those of authentic standards or

188   published values.

189

190

191   **Headspace solid-phase microextraction**

192   We carried out volatile compound analysis by automated headspace solid-phase microextraction

193   (SPME), followed by GC–MS, using an Agilent 110 PAL injection system and Agilent 7890 gas

194   chromatograph with 5975C mass spectrometer. The SPME fibre stationary phase was composed

195   of 75 μm divinylbenzene/Carboxen™ on polydimethylsiloxane (Supelco, Bellefonte, PA). The

196   uncapped 2-mL vials containing the MCT extracts were placed in 20-mL headspace vials. The

197   samples were then equilibrated for 10 minutes at 37 °C before being extracted for 30 min.

198   Samples were agitated at 500 rpm (5 s on, 2 s off) during equilibration and extraction. After

199   extraction, the contents of the fibre were desorbed in splitless mode at 250 °C onto the front of a

200   Stabilwax DA fused silica capillary column (30 m, 0.25 mm i.d., 0.50 μm film thickness; Restek,

201   Bellefonte PA). The GC temperature program and the fibre desorption step commenced at the

202   same time. During the desorption period of 45 s, the oven was held at 40 °C. After desorption,

203   the oven was held at 40 °C for a further 255 s before heating at 4 °C/min to 260 °C, where the

204   temperature was maintained for 5 min. Helium was used as the carrier gas at a constant flow rate

205   of 0.9 mL/min. A series of *n*-alkanes (C_5–C_{22}) in diethyl ether was analysed, under the same

206   conditions, to obtain LRI values for the components of the extract. To check for the presence of

207   volatiles contributed from the MCT solvent, we ran a blank extraction of the MCT alone within

208   the sample vial. We detected some octanoic acid (this was predicted, as the MCT used was

209   glyceryl trioctanoate), that we note in the results.

210   The mass spectrometer operated in electron impact mode with an electron energy of 70

211   eV, scanning from *m/z* 20 to *m/z* 280 at 1.9 scans/s. As with the direct injection protocol, we

212   identified compounds by first comparing their mass spectra with those contained in the

213   NIST/EPA/NIH Mass Spectral Database or in previously published literature. Wherever possible

214   we then confirmed identities by comparison of linear retention index (LRI) values with those of

215   authentic standards.

216

217   **Chemical confirmation of compounds identified**

218   Authentic standards for confirmation of identities and/or synthesis included 3,4-

219   dimethoxybenzoic acid (veratric acid) (I), 4-(4-methoxyphenyl)butyric acid (II), β-(4-hydroxy-3-

220   methoxyphenyl)propionic acid (III), 3-(3,4-dimethoxyphenyl)propionic acid (IV), linalool oxide

221   (pyranoid), 3,4-dimethylbenzaldehyde, 1-methoxy-2-propanol, 1-propanol and acrylic acid, all

222   purchased from Tokyo Chemical Industry UK, Ltd (Oxford, UK); 3-(3,5-dimethoxy-4-

223   hydroxyphenyl)propionic acid (V) was purchased from Carbosynth (Compton, UK); β-(3-

224   hydroxy-4-methoxyphenyl)propionic acid (VI), anthranilic acid (VII), vanillic acid (VIII),

225   octanoic acid (IX), salicylic acid (X) and propylene glycol were obtained from Sigma-Aldrich

226   (Gillingham, Dorset, UK).

227   To confirm the identities of tentatively identified propyl and hydroxypropyl esters, we

228   performed synthesis using acids I to X above. Ten reactions were carried out in 1-mL Reacti-

229   Vials (Thermo Fisher Scientific). Each Reacti-Vial contained 0.2 g of acid and 0.2–0.6 mL of a

230   1:1 mixture of 1-propanol and propylene glycol plus 1 drop of hydrochloric acid as catalyst. The

231   amount of alcohol added depended on the solubility of the acid. Reaction mixtures were heated

232   for 30 min at 80 °C and then neutralised by dropwise addition of 0.1 M sodium bicarbonate. The

233   esters were then transferred to 7-mL glass bottles and 2 mL of diethyl ether were added. The

234   contents of the bottles were then stirred at 700 rpm using a magnetic stirrer for 5 minutes. The

235   contents were allowed to settle and the ether layer was removed for analysis by GC–MS, after

236   addition of a small amount of anhydrous sodium sulfate to remove any water still present. The

237   GC–MS conditions used were the same as those used for the direct injection of the MCT

238   extracts, only with a split ratio of 100:1.

239

240   **Statistical analysis**

241   We identified GC peaks and calculated their retention index and peak areas using the software

242   MSD Chemstation E02.02.1431 (2011; Agilent, Santa Clara, CA). For direct injection and

243   headspace analyses, we compared both mean peak areas from the GC output and the relative

244   peak area (%) of each compound to the total peak area (of all peaks we identified) among adult

245   males (of reproductive age), subadult/juvenile males (non-reproductive), and adult females.

246   Absolute mean peak areas give an indication of quantitative differences between samples and

247   groups but can be subject to variance in sampling. The percentage peak areas normalise the data

248   across samples, and correct for differences in absolute amounts of starting sample (due to the

249   difficulties in taking an equivalent amount of fur and secretion from each bat). In our analyses,

250   we examined both mean and relative (%) peak areas. Taking an approach used by Schneeberger

251   *et al.* (2016), we compared the overall chemical compositions (the "odour fingerprint") of each

252   individual sample. A Bray–Curtis dissimilarity index was then computed using adult males,

253   juvenile males and adult females as categories for prediction in a non-parametric analysis of

254   similarities (ANOSIM) and to identify significant differences among groups. We visualised data

255   in two dimensions with a non-metric multidimensional scaling (NMDS) ordination. Similarity

256   percentage (SIMPER) was then used to identify significant differences between equivalent peaks

257   in each sample, and, hence, which odour components contributed most to the differences

258   between groups. We performed all analyses using the VEGAN package in RStudio (version

259   1.0.136), and both the ANOSIM and SIMPER procedures used 1000 permutations.

260

261   **Results**

262   We identified 15 compounds in medium-chain triglyceride extracts of fur surrounding the

263   shoulder gland of *Sturnira parvidens*, some of which differed significantly between adult males

264   and juvenile males/adult females (n=5 samples from each age/sex class; Table 1; Supplementary

265   Figure 1). Three of the 15 compounds occurred only in adult males, namely vanillic acid, 3,4-

266   dimethoxybenzoic acid (veratric acid), and 3-(3,4-dimethoxyphenyl)propionic acid. We found

267   two other compounds in both adult males and females (levels significantly higher in males), but

268   not juvenile males: hydroisoferulic acid propyl ester and 3-(3,5-dimethoxy-4-

269   hydroxyphenyl)propionic acid propyl ester. Four additional compounds showed significant

270   differences between the three groups, and in all cases the adult males had higher levels of these

271   compounds, while juvenile males did not differ from females. Of these four, α,β-dihydroferulic

272   acid propyl ester had the greatest peak area; the other compounds were the propyl ester of

273   anthranilic acid, 4-(4-methoxyphenyl)butyric acid, the propyl ester of α,β-dihydroferulic acid,

274   and the hydroxypropyl ester of α,β-dihydroferulic acid. A single compound, β-(4-hydroxy-3-

275   methoxyphenyl)propionic acid (α,β-dihydroferulic acid), was found in all groups and while

276   higher in adult males, only reached a significant difference in adult males versus adult females.

277   Supplementary Table 1 provides supporting mass spectral data of the fifteen compounds

278   identified in the medium-chain triglyceride extracts of fur.

279   We undertook headspace concentration of the solvent extract, to discover if there were

280   compounds of interest present at low concentrations in the fur extract. Table 2 lists the headspace

281   compounds in medium-chain triglyceride extracts of fur surrounding the shoulder gland of

282   *Sturnira parvidens*, obtained using solid-phase microextraction, and representative

283   chromatograms are shown in Supplementary Figure 2. These are the more volatile components in

284   the extracted glandular secretion from *Sturnira parvidens* shoulder gland fur. We identified 42

285   compounds, 33 showing significant differences between adult males, juvenile males and adult

286   females for peak area (6 compounds), percentage peak area (14 compounds) or both (13

287   compounds). Twenty-one peaks were higher and eleven peaks were lower in adult males,

288   compared to females and juveniles. One peak was higher in females versus juvenile males for

289   percentage peak area (acrylic acid). Collectively these results indicate that adult male shoulder

290   gland secretion odour profiles are distinct from those of females and juvenile males. The twelve

291   peaks with the largest area that were significantly greater in males compared to females and

292   juvenile males were 1-propanol, acetic acid, 2-butanol, guaiacol, anthranilic acid propyl ester,

293   (*Z*)-linalool oxide furanoid, phenol, 3-methylpyridine, propyl salicylate, 4-vinyl-2-

294   methoxyphenol, (*E*) *or* (*Z*)-linalool oxide (pyranoid), and 4-ethyl-2-methoxyphenol, respectively.

295   Linalool was approximately eight times higher in males (mean peak area) than females and

296   juvenile males but was only significantly different versus females.

297   Figure 2 shows the overall odour fingerprint analysis as non-metric multidimensional

298   scaling (NMDS) plots for direct injection and headspace extracts, and for both the absolute and

299   relative percentage peak areas. In all cases, adult male profiles were distinct from those of adult

300   females and juvenile males, with the latter showing an overlapping pattern. Multivariate

301   statistical analysis of similarity of the relationship between grouping variable (adult and juvenile

302   males and adult females) and chemical composition of fur extract showed a significant effect of

303   group (Headspace ANOSIM on Bray–Curtis similarity matrix, R=0.4922, P≤0.001, Direct

304   Injection ANOSIM on Bray–Curtis similarity matrix, R=0.5911, P≤0.001).

305

Discussion

307   Odour fingerprint analysis of extracts of fur surrounding the shoulder gland of *Sturnira*

308   *parvidens*, using multivariate analysis, revealed clear, significant differences between adult

309   males versus both juvenile males and adult females. Many of the compounds identified included

310   terpenes and phenolics, together with alcohols and esters, and occurred exclusively or in elevated

311   quantities among adult (reproductive) males, compared with adult females and non-reproductive

312   sub-adult/juvenile males. This strongly suggests a specific role in male–female attraction for

313   these compounds, although a function in male-male competition and/or species recognition is

314   also possible. While non-reproductive responses have been attributed to pheromones (e.g.,

315   aggression and alarm signals), sexual stimulation and attraction are the most commonly

316   associated traits of pheromones (though the two responses can happen simultaneously to the

317   same substance for different individuals (Novotny, 2003)).

318   Among animals where vision is not a primary sensory modality, chemical cues have been

319   associated with species recognition. Many species of bat are highly gregarious, forming large,

320   often multi-species colonies that are stable units (Kunz 1982), and chemical recognition is

321   known to assist in mother–pup recognition (De Fanis & Jones 1996) and colony identification

322   (Bloss et al. 2002). Although relatively little is known about *Sturnira* social systems, they are not

323   known to roost in the large groups or colonies typical of many bats, but rather in small groups in

324   tree holes or other small cavities (Fenton et al., 2000). However, the distinctiveness of the

325   shoulder gland odour profiles that we have characterised for reproductively active adult males

326   suggests strongly that they are more likely to be involved in mating or mate choice. A small

327   number of other studies among bats have suggested a role for odour communication in individual

328   and social group recognition. Female big brown bats (*Eptesicus fuscus*) were able to use

329   chemical cues to distinguish roost mates and in a Y-maze experiment chose to preferentially

330   associate with the familiar cue (Bloss et al., 2002). In female Bechstein's bats, *Myotis*

331   *bechsteinii*, odour profiles from secretions of the facial interaural gland are individually specific

332   and differ between colonies, suggesting a function in individual and colony recognition (Safi &

333   Kirth, 2003). A study of the distinctive odor of the fisherman bat (*Noctilio leporinus*), produced

334   by a secretion from glands in the sub-axillary region beneath the wings, identified 372 lipid

335   compounds (Brooke & Decker, 1996). Differences in the chemical composition suggested that

336   secretions of males from the same roost were more similar to each other than to other males or

337   females. Furthermore, as secretions differed between the sexes, information on sexual identity

338   and reproductive condition could potentially be communicated. As with *Sturnira parvidens*,

339   another phyllostomid, the male greater spear-nosed bat, *Phyllostomus hastatus*, also possesses a

340   sexually dimorphic gland that produces an odoriferous secretion, but it is found on the chest

341   rather than the shoulder. A recent study by Adams et al., (2018) reports that secretions from male

342   harem holders, that defend and roost with groups of females, had significantly different chemical

343   profiles from bachelor males found roosting in all male groups. Odour profiles also differed

344   significantly among individuals, suggesting that the chemical signal has the potential to

345   communicate both mating status and individual identity.

346   While the aforementioned provide good evidence for odour signals/pheromones playing

347   an important role in reproductive and social behaviour in bats, our study goes further in

348   identifying the chemical composition of the *Sturnira parvidens* odour signal. In the adult male

349   shoulder gland secretion, the components include terpenes and phenolics, together with alcohols

350   and esters. It is likely that these are derived from the frugivorous diet of *Sturnira parvidens*.

351   Bloss et al. (2002) identified 15 potential chemicals associated with female *Eptesicus fuscus* and,

352   of these, linalool was also found in our analysis of *Sturnira parvidens*, together with three

353   linalool derivatives, all of which were elevated in reproductive males. Linalool is a terpene

354   alcohol naturally produced by over 200 plant species, and there are many examples of it

355   functioning to attract insects to plants (e.g., hawkmoths; Raguso and Light, 1998), and is the

356   mate attractant pheromone in the bee *Colletes cunicularius* (Borg-Karlson et al., 2003),

357   suggesting a very broad biological action across plant and animal kingdoms.

358   Phenolics found in higher concentrations in the adult male secretions in our analysis

359   include vanillic acid (a natural phenol found, for example, in palm fruit), and guaiacol. The latter

360   is a component of the pheromone that causes locust swarming (Dillon et al., 2000) and produced

361   in the gut of desert locusts, *Schistocerca gregaria*, by the breakdown of plant material. Phenol

362   itself was also identified as a major component in our adult male samples, and interestingly is

363   also part of the temporal gland secretion of adult male elephants, which is thought to function to

364   attract females during muste, the periodic aggressive behavioural condition exhibited by bull

365   elephants (Rasmussen & Perrin, 1999). We also identified esters of ferulic acid, the latter being a

366   phenolic antioxidant found in the seeds of apples and oranges and the cell walls of many plants.

367   In addition to vanillic acid, we identified a number of other phenolic acids and propyl

368   esters derived from them (Supplementary Table 2). These included dihydroferulic,

369   hydroisoferulic, hydrosinapinic, veratric and anthranilic propyl esters and the hydroxypropyl

370   esters of dihydroferulic acid and anthranilic acid (Table 1). Interestingly, α,β-dihydroferulic acid

371   propyl ester has been shown to exhibit strong antifungal action, perhaps suggesting a role beyond

372   just odour signalling for this compound (Beck et al., 2007). Significant quantities of 1-propanol

373   were found in the headspace analysis of the adult male compared to other alcohols. By contrast,

374   only small quantities of 1-propanol were found in the headspace of the juvenile male and the

375   female, suggesting that it and/or the propyl esters may play a role in pheromonal signalling in

376   this bat species. 1-Propanol may either be acting as a chemical signal in its own right, or

377   produced to form the aforementioned esters with the various phenolic acids. All the acids and

378   esters found in the adult male extract were at significantly higher levels than in the juvenile male

379   and the female. Additionally, anthranilic acid propyl ester was also identified in the headspace

380   analysis (Table 2) at significantly higher levels in the adult male than in the juvenile male and

381   the female. The hydroxypropyl esters of dihydroferulic acid and anthranilic are derived from

382   propylene glycol, which was also identified in significantly higher quantities in the headspace

383   analysis of the adult male, compared to the juvenile male and the female, suggesting that these

384   esters may also have pheromonal or semiochemical activity. Three of these esters (Table 2) have

385   not been previously reported and are unique to *Sturnira parvidens*.

386   Bacteria have been associated with the production of many chemical cues and it is

387   possible that these may also play a role in the synthesis of some of the compounds that we

388   identified. In turn, these may contribute to individual and population-specific odour signatures in

389   *Sturnira parvidens* (if populations share local communities of bacteria in their shoulder glands).

390   In hyenas, bacterial species co-vary with odour profiles specific to populations, supporting such

391   a "fermentation hypothesis" of bacterial mediated chemical communication (Theis et al., 2013).

392   An investigation of bacteria associated with the epaulettes (shoulder glands) of the related bat

393   species *Sturnira lilium* and *S. bogotensis* revealed few common bacterial species between males

394   and females, offering the possibility for sex-specific odour production in the glands of males

395   (Gonzalez Quinonez et al., 2014).

396   **Conclusions**

397   In summary, many of the compounds we identified from our analysis were found exclusively or

398   in elevated quantities among adult (reproductive) males, compared with adult females and non-

399   reproductive males. Similar analyses of other *Sturnira parvidens* populations, and other *Sturnira*

400   species would be interesting, enabling a comparison of the variance in the composition of the

401   odour signal. Our results strongly suggests a specific role in male–female attraction in this

402   species, but further behavioural work is needed to confirm the functional significance of the

403   adult male shoulder gland secretions in *Sturnira parvidens*.

404

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409

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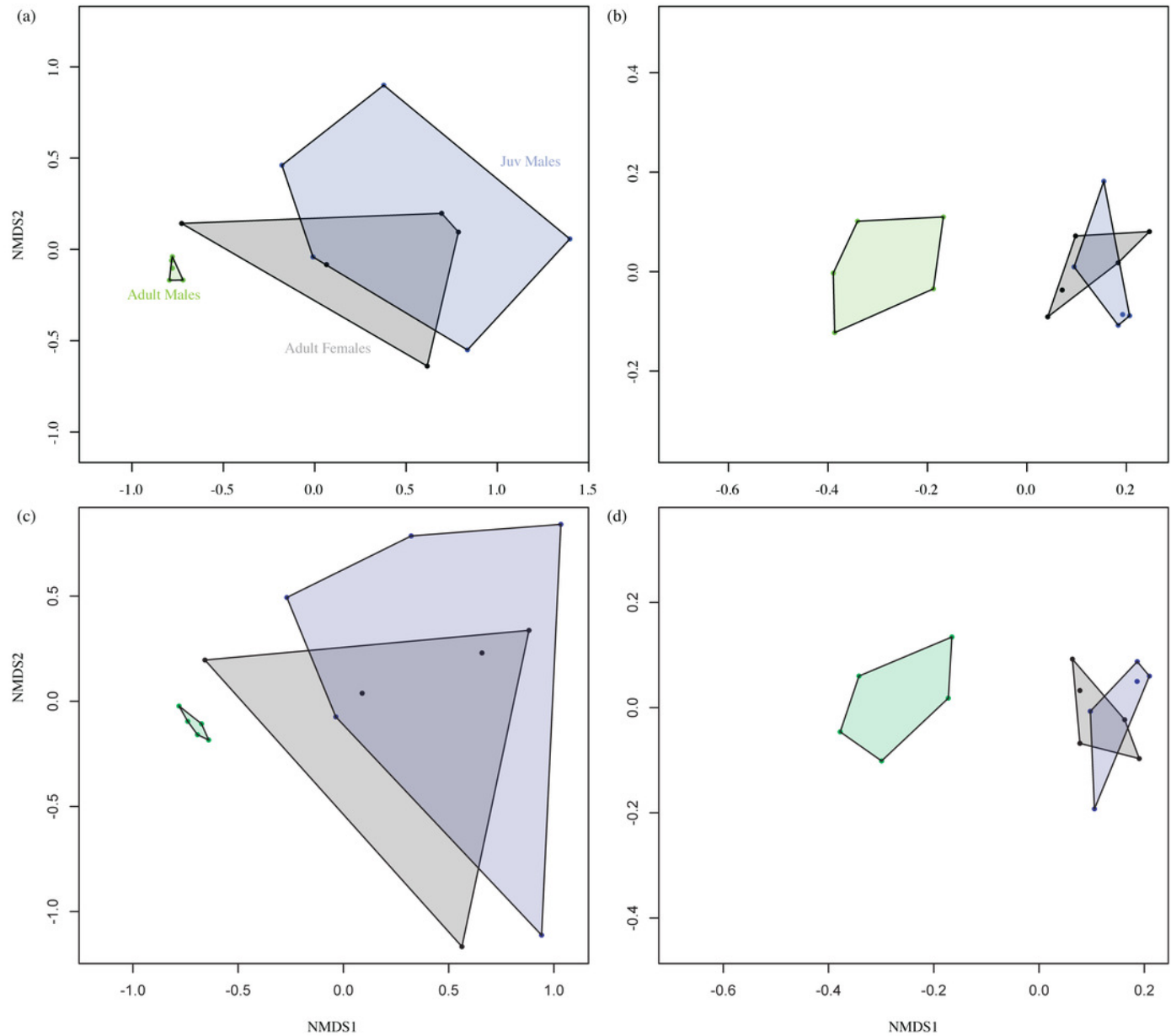
524

Figure 1

Sturnira parvidens adult male showing dark staining of fur surrounding the shoulder gland (Photo credit: Brock Fenton).

Figure 2

Non-metric multidimensional scaling (NMDS) plot.

Data represents the overall odour profiles of (a) and (c) direct injection, (b) and (d) headspace extracts from the shoulder gland secretions of adult males, adult females and juvenile (Juv) males. Profiles (a) and (b) are generated from the relative percentage peak area, while (c) and (d) are from absolute peak areas. Adult male profiles are distinct from those of females and juveniles, which show an overlapping pattern. Axes are dimensionless and have no units.

(a)

NMDS2

Juv Males

Adult Males

Adult Females

(b)

(c)

NMDS2

NMDS1

(d)

NMDS1

Compounds in medium-chain triglyceride extracts of fur surrounding the shoulder gland of *Sturnira parvidens*.

Data are mean chromatographic peak areas (standard deviation in parentheses), together with relative peak areas expressed as a mean % of the total across compounds (shown in italics). All identifications were confirmed with reference to standard compounds. LRI, linear retention index on a 15 m × 0.25 mm (0.25 µm film thickness) DB-5MS capillary column. *p*= probability of a significant difference; Peak areas followed by the same letter are not significantly different (*p*> 0.05), while peak numbers refer to Supplementary Figure 1.

Peak number	LRI	compound	gas chromatographic peak areas ($\times 10^{-6}$) *relative peak areas (%)*			p
			adult male	juvenile male	adult female	
1	1386	anthranilic acid	3.95 (1.78)	0.161 (0.212)	0.153 (0.262)	NS
			4.92 (3.73)	*10.92 (12.93)*	*5.09 (7.59)*	NS
2	1503	anthranilic acid propyl ester	14.7 (11.4)**b**	0.214 (0.234)**a**	0.186 (0.126)**a**	<0.05
			11.86 (3.28)	*15.59 (11.21)*	*18.69 (7.98)*	NS
3	1552	vanillic acid	0.429 (0.061)	—	—	<0.001
			0.72 (0.70)	—	—	<0.0001
4	1626	3,4-dimethoxybenzoic acid (veratric acid)	1.46 (0.945)	—	—	<0.001
			1.43 (0.67)	—	—	<0.0001
5	1666	4-(4-methoxyphenyl)butyric acid	12.6 (16.8) **b**	0.0107 (0.0239)**a**	0.0335 (0.0749)**a**	<0.001
			*6.78 (3.07)**b***	*0.67 (1.50)**a***	*0.94 (2.10)**a***	<0.01
6	1671	vanillic acid propyl ester	5.15 (6.42)	0.0058 (0.0129)	0.0190 (0.0426)	NS
			2.18 (2.11)	*0.82 (1.83)*	*0.53 (1.19)*	NS
7	1682	anthranilic acid hydroxypropyl ester	3.30 (2.54)	0.0570 (0.109)	0.054 (0.0741)	NS
			2.48 (0.57)	*2.38 (4.19)*	*2.14 (3.09)*	NS
8	1722	β-(4-hydroxy-3-methoxyphenyl)propionic acid (α,β-dihydroferulic acid)	3.73 (2.77)**a**	0.298 (0.262)**ab**	0.172 (0.174)**b**	<0.05
			3.44 (1.51)	*34.56 (33.65)*	*2.14 (3.09)*	NS
9	1741	β-(3-hydroxy-4-methoxyphenyl)propionic acid (hydroisoferulic acid)	0.802 (0.648)	0.0147 (0.0223)	0.0049 (0.0108)	NS
			0.72 (0.41)	*0.81 (1.37)*	*1.26 (2.81)*	NS
10	1744	veratric acid propyl ester	0.401 (0.233)	0.0063 (0.0140)	0.0155 (0.0346)	NS
			0.42 (0.24)	*0.39 (0.88)*	*0.43 (0.97)*	NS
11	1764	3-(3,4-dimethoxyphenyl)propionic acid	0.711 (0.621)	—	—	<0.001
			0.54 (0.16)	—	—	<0.0001
12	1828	α,β-dihydroferulic acid, propyl ester	96.3 (95.7)**b**	0.427 (0.416)**a**	0.688 (0.712)**a**	<0.001
			*60.21 (16.39)**a***	*33.38 (26.14)**b***	*49.43 (11.71)**ab***	<0.06
13	1852	hydroisoferulic acid, propyl ester	3.16 (3.19)**b**	—	0.0233 (0.0412)**a**	<0.01
			2.03 (0.74)	—	*2.35 (3.94)*	*NS*
14	1984	α,β-dihydroferulic acid, hydroxypropyl ester	2.62 (2.47)**b**	0.0121 (0.0270)**a**	0.0297 (0.0425)**a**	<0.05
			1.64 (0.43)	*0.47 (1.05)*	*1.11 (1.53)*	NS
15	2055	3-(3,5-dimethoxy-4-hydroxyphenyl)propionic acid propyl ester (hydrosinapinic acid propyl ester)	1.14 (1.48)**b**	—	0.0083 (0.0185)**a**	<0.05
			*0.63 (0.37)**b***	—	*0.23 (0.52)**a***	<0.05

Headspace compounds in medium-chain triglyceride extracts of fur surrounding the shoulder gland of *Sturnira parvidens*, obtained using solid-phase microextraction.

Data are mean chromatographic peak areas (standard deviation in parentheses), together with relative peak areas expressed as a mean % of the total across compounds (shown in italics). *p*= probability of a significant difference; Peak areas followed by the same letter are not significantly different (*p*> 0.05)), while peak numbers refer to Supplementary Figure 2.

Peak number	linear retention index (LRI)[a]	compound	confirmation of identity[b]	gas chromatographic peak areas ($\times 10^{-6}$)			
				adult male	juvenile male	adult female	p
1	904	ethyl acetate	Std	93.9 (17.9)**a**	120 (10.8)**b**	126 (9.31)**b**	0.006
				*6.46 (2.27)**a***	*17.86 (2.83)**b***	*17.89 (1.85)**b***	*<0.01*
2	912	isopropyl acetate	Std	41.4 (9.89)**a**	51.2 (7.70)**ab**	55.3 (4.87)**b**	0.041
				*2.85 (1.08)**a***	*7.64 (1.39)**b***	*7.85 (0.60)**b***	*<0.05*
3	916	2-butanone	Std	52.6 (18.6)	61.6 (10.7)	67.9 (9.64)	NS
				*3.67 (1.81)**a***	*9.19 (1.92)**b***	*9.60 (0.87)**b***	*<0.05*
4	939	isopropyl alcohol	Std	76.4 (23.0)	90.7 (8.95)	96.8 (6.14)	NS
				*5.52 (3.55)**a***	*13.72 (3.35)**b***	*13.93 (2.50)**a***	*<0.05*
5	947	ethanol	Std	115 (47.8)	162 (43.5)	154 (22.6)	NS
				*7.40 (1.95)**a***	*24.56 (8.64)**b***	*22.14 (5.13)**b***	*<0.05*
6	972	ethyl propanoate	Std	9.78 (2.36)	9.91 (1.95)	10.7 (2.47)	NS
				*0.68 (0.28)**a***	*1.47 (0.30)**b***	*1.57 (0.54)**b***	*<0.05*
7	1030	alpha-pinene	Std	8.81 (0.994)	12.6 (8.36)	12.8 (12.2)	NS
				0.60 (0.17)	*1.96 (1.57)*	*1.65 (1.31)*	*NS*
8	1038	2-butanol	Std	17.0 (4.15)**b**	9.02 (1.15)**a**	7.06 (3.50)**a**	0.001
				1.13 (0.26)	*1.34 (0.22)*	*0.98 (0.49)*	*NS*
9	1052	1-propanol	Std	674 (114)**b**	44.4 (73.7)**a**	50.0 (65.4)**a**	<0.0001
				*44.58 (8.12)**b***	*5.90 (9.32)**a***	*6.23 (7.09)**a***	*<0.01*
10	1096	*N,N*-dimethylhydroxylamine	MS	10.3 (9.53)	4.40 (5.63)	11.8 (10.9)	NS
				4.79 (9.17)	*0.52 (0.65)*	*1.43 (1.15)*	*NS*
11	1132	2-pentanol	Std	2.28 (1.35)**b**	0.776 (0.078)**a**	0.956 (0.156)**ab**	0.021
				0.14 (0.04)	*0.12 (0.01)*	*0.14 (0.02)*	*NS*
12	1138	3-methylthiophene	Std	2.66 (2.25)	5.83 (10.9)	1.32 (1.45)	NS
				0.15 (0.11)	*0.73 (1.34)*	*0.98 (0.49)*	*NS*
13	1140	1-methoxy-2-propanol	Std	7.64 (4.21)	8.36 (5.27)	10.6 (3.71)	NS
				*0.47 (0.16)**a***	*1.25 (0.85)**ab***	*1.49 (0.52)**b***	*<0.05*
14	1156	1-butanol	Std	9.55 (6.42)	4.66 (0.587)	4.32 (1.03)	NS
				*0.58 (0.22)**a***	*0.69 (0.10)**b***	*0.61 (0.11)**ab***	*<0.05*
15	1212	limonene	Std	5.19 (2.00)	3.85 (3.25)	3.68 (1.44)	NS
				0.33 (0.14)	*0.54 (0.37)*	*0.53 (0.22)*	*NS*
16	1262	3-methyl-3-buten-1-ol	Std	3.95 (3.15)	3.28 (3.79)	11.7 (15.7)	NS
				*0.25 (0.14)**a***	*0.43 (0.47)**ab***	*1.46 (1.69)**b***	*<0.05*

17	1313	3-methylpyridine	Std	1.17 (0.619)**b**	0.248 (0.156)**a**	0.403 (0.135)**a**	0.005
				*0.08 (0.04)**a***	*0.03 (0.02)**b***	*0.06 (0.02)**ab***	*<0.05*
18	1404	methyl octanoate	Std	5.51 (5.69)	6.18 (10.4)	5.53 (5.17)	NS
				0.29 (0.24)	*0.79 (1.26)*	*0.79 (1.26)*	*NS*
19	1448	ethyl octanoate	Std	3.04 (2.70)	3.58 (4.77)	4.84 (4.56)	NS
				0.17 (0.11)	*0.47 (0.57)*	*0.69 (0.65)*	*NS*
20	1457	(*E*)-linalool oxide furanoid	Std	0.974 (1.08)	0.011 (0.024)	0.042 (0.040)	0.05
				*0.05 (0.05)**b***	*<0.00 (0.00)**a***	*0.01 (0.01)**a***	*<0.01*
21	1474	acetic acid	Std	38.9 (14.7)**b**	15.1 (7.80)**a**	17.9 (3.65)**a**	0.005
				2.68 (1.41)	*2.15 (0.77)*	*2.56 (0.57)*	*NS*
22	1485	(*Z*)-linalool oxide furanoid	Std	3.98 (3.92)**b**	0.043 (0.043)**a**	0.080 (0.079)**a**	0.026
				*0.24 (0.23)**b***	*0.01 (0.01)**a***	*0.01 (0.01)**a***	*<0.01*
23	1533	propyl octanoate	Std	21.6 (31.2)	0.360 (0.328)	0.458 (0.400)	NS
				*1.07 (1.45)**b***	*0.05 (0.04)**a***	*0.06 (0.06)**a***	*<0.01*
24	1551	benzaldehyde	Std	2.32 (1.27)	1.45 (0.167)	1.54 (0.187)	NS
				*0.16 (0.11)**a***	*0.21 (0.02)**b***	*0.22 (0.04)**b***	*<0.01*
25	1558	linalool	Std	0.855 (0.753)**b**	0.127 (0.029)**ab**	0.104 (0.062)**a**	0.029
				0.05 (0.04)	*0.02 (0.01)*	*0.02 (0.01)*	*NS*
26	1560	propanoic acid	Std	2.83 (0.843)**b**	1.61 (0.753)**a**	1.852 (0.318)**ab**	0.034
				0.19 (0.06)	*0.23 (0.07)*	*0.27 (0.06)*	*NS*
27	1620	propylene glycol	Std	41.4 (39.3)**b**	2.46 (0.598)**a**	4.38 (1.24)**ab**	0.032
				*2.68 (2.74)**b***	*0.36 (0.06)**a***	*0.63 (0.21)**a***	*<0.01*
28	1648	butyric acid	Std	3.62 (2.13)	1.51 (0.967)	2.31 (1.32)	NS
				0.24 (0.15)	*0.21 (0.11)*	*0.33 (0.19)*	*NS*
29	1663	acrylic acid	Std	9.29 (3.74)	2.78 (3.58)	7.40 (4.88)	NS
				*0.59 (0.12)**ab***	*0.37 (0.42)**a***	*1.01 (0.57)**b***	*<0.01*
30	1757	pentanoic acid	Std	1.44 (1.27)	0.527 (0.293)	0.722 (0.263)	NS
				0.10 (0.11)	*0.07 (0.03)*	*0.10 (0.04)*	*NS*
31	1777	(*E*)- or (*Z*)-linalool oxide (pyranoid)	Std	0.926 (0.808)**b**	0.006 (0.013)**a**	0.025 (0.035)**a**	0.013
				*0.05 (0.04)**b***	*<0.00 (0.00)**a***	*<0.00 (0.00)**a***	*<0.01*
32	1847	3,4-dimethylbenzaldehyde	Std	1.85 (0.362)	2.16 (0.506)	2.01 (0.421)	NS
				*0.13 (0.04)**a***	*0.32 (0.06)**b***	*0.29 (0.07)**b***	*<0.01*
33	1864	hexanoic acid	Std	4.04 (1.43)**b**	1.68 (1.13)**a**	1.80 (0.880)**a**	0.012
				0.26 (0.07)	*0.23 (0.12)*	*0.26 (0.13)*	*NS*
34	1886	guaiacol	Std	8.19 (7.91)**b**	0.075 (0.029)**a**	0.116 (0.063)**a**	0.023
				*0.50 (0.54)**b***	*0.01 (0.00)**a***	*0.02 (0.01)**a***	*<0.01*
35	1899	benzyl alcohol	Std	5.85 (8.05)	0.210 (0.045)	0.704 (0.502)	NS
				*0.44 (0.67)**b***	*0.03 (0.00)**a***	*0.10 (0.07)**ab***	*<0.05*
36	1925	propyl salicylate	Std	1.02 (0.707)**b**	0.024 (0.018)**a**	0.027 (0.015)**a**	0.003
				*0.07 (0.06)**b***	*<0.00 (0.00)**a***	*<0.00 (0.00)**a***	*<0.01*

37	2031	phenol	Std	1.48 (0.382)**b**	0.326 (0.089)**a**	0.772 (0.252)**a**	0.0001
				*0.01 (0.03)**ab***	*0.05 (0.01)**a***	*0.11 (0.04)**b***	*<0.01*
38	2056	5-ethyl-2-methoxyphenol	MS + LRI	0.742 (0.563)**b**	0.032 (0.023)**a**	0.025 (0.006)**a**	0.006
				*0.04 (0.02)**b***	*<0.00 (0.00)**a***	*<0.00 (0.00)**a***	*<0.01*
39	2061	4-ethyl-2-methoxyphenol	Std	0.551 (0.639)	0.019 (0.011)	0.023 (0.009)	NS
				*0.03 (0.03)**b***	*<0.00 (0.00)**a***	*<0.00 (0.00)**a***	*<0.01*
40	2076	octanoic acid*	Std	200 (302)	49.8 (81.0)	32.9 (28.7)	NS
				9.78 (14.14)	*6.43 (9.76)*	*4.71 (4.17)*	*NS*
41	2197	4-vinyl-2-methoxyphenol	Std	1.01 (0.635)**b**	0.032 (0.025)**a**	0.038 (0.022)**a**	0.002
				*0.06 (0.03)**b***	*<0.00 (0.00)**a***	*0.01 (0.00)**a***	*<0.01*
42	>2200	anthranilic acid propyl ester	Std	5.71 (4.55)**b**	0.150 (0.054)**a**	0.222 (0.124)**a**	0.008
				*0.35 (0.30)**b***	*0.02 (0.01)**a***	*0.03 (0.02)**a***	*<0.01*

3   [a] Linear retention index on a 30 m × 0.25 mm (0.5 μm film thickness) Stabilwax DA capillary column
4   [b] Std: standard compound run under the same conditions; MS + LRI: mass spectrum and linear retention index similar to literature spectrum (Schranz et al.,
5   2017); MS: mass spectrum similar to literature spectrum (NIST11.L for Chemstation)
6   *octanoic acid is also contributed by breakdown of the MCT solvent.