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1 **Cuelure but not zingerone make the sex pheromone of male *Bactrocera tryoni***

2 **(Tephritidae: Diptera) more attractive to females**

3 Nagalingam Kumaran[1 *], R Andrew Hayes[2, 3] and Anthony R Clarke[1, 4]

4 [1]School of Earth, Environmental and Biological Sciences, Queensland University of

5 Technology, Brisbane, Australia

6 [2]Agri-Science Queensland, Department of Agriculture, Fisheries and Forestry,

7 Ecosciences Precinct, Dutton Park, Qld 4102, Australia

8 [3]Forest Industries Research Centre, University of the Sunshine Coast, Sippy Downs,

9 Qld 4558, Australia

10 [4]Plant Biosecurity Cooperative Research Centre, Bruce, ACT, Australia

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12 ***Corresponding author**

13 Nagalingam Kumaran

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21 **Abstract**

22 In tephritid fruit flies of the genus *Bactrocera* Macquart, a group of plant derived
23 compounds (*sensu amplo* 'male lures') enhance the mating success of males that have
24 consumed them. For flies responding to the male lure methyl eugenol, this is due to
25 the accumulation of chemicals derived from the male lure in the male rectal gland
26 (site of pheromone synthesis) and the subsequent release of an attractive pheromone.
27 Cuelure, raspberry ketone and zingerone are a second, related group of male lures to
28 which many *Bactrocera* species respond. Raspberry ketone and cuelure are both
29 known to accumulate in the rectal gland of males as raspberry ketone, but it is not
30 known if the emitted male pheromone is subsequently altered in complexity or is
31 more attractive to females. Using *Bactrocera tryoni* as our test insect, and cuelure and
32 zingerone as our test chemicals, we assess: (i) lure accumulation in the rectal gland;
33 (ii) if the lures are released exclusively in association with the male pheromone; and
34 (iii) if the pheromone of lure-fed males is more attractive to females than the
35 pheromone of lure-unfed males. As previously documented, we found cue-lure was
36 stored in its hydroxyl form of raspberry ketone, while zingerone was stored largely in
37 an unaltered state. Small but consistent amounts of raspberry ketone and β-(4-
38 hydroxy-3-methoxyphenyl)-propionic acid were also detected in zingerone-fed flies.
39 Males released the ingested lures or their analogues, along with endogenous
40 pheromone chemicals, only during the dusk courtship period. More females
41 responded to squashed rectal glands extracted from flies fed on cuelure than to glands
42 from control flies, while more females responded to the pheromone of calling cuelure-
43 fed males than to control males. The response to zingerone treatments in both cases
44 was not different from the control. The results show that male *B. tryoni* release

45 ingested lures as part of their pheromone blend and, at least for cuelure, this attracts
46 more females.

47 **Key Words** - Male lures, mating, courtship, methyl eugenol, raspberry ketone, mate
48 selection, Dacinae, fruit fly, female choice.

49

1. Introduction

The production and release of sex pheromones is a key part of the mating system of many insects. While many pheromones are fully produced intrinsically (Tillman et al., 1999), pheromone production in some insects is driven or enhanced by the consumption of secondary plant metabolites from host or non-host plants (Beyaert and Hilker, 2013; Krasnoff and Dussourd, 1989; Landolt and Phillips, 1997; Reddy and Guerrero, 2004). For example, volatile chemicals from corn silk triggers the production and release of sex pheromone in *Helicoverpa zea* (Boddie) (Raina et al., 1992), while the arctiid moths *Creatonotos gangis* (L.) and *C. transiens* (Walker) release significantly altered pheromones after feeding as larvae on plants containing pyrolizidine alkaloids (Schneider et al., 1975).

In the dipteran family Tephritidae, the males of many *Bactrocera* Macquart species have an association with plant secondary metabolites that influence male mating advantage, presumed to be due to the altering of the male pheromone composition (Raghu, 2004; Shelly, 2010). These phytochemicals include 1, 2-dimethoxy-4-(2-propenyl)-benzene (methyl eugenol - ME), 4-(4-hydroxyphenyl)-2-butanone (raspberry ketone) and 4-(4-hydroxy-3-methoxyphenyl)-2-butanone (zingerone), which are found as secondary chemicals in a wide range of plant families (Metcalf and Metcalf, 1992). Very commonly, these plants are not hosts of *Bactrocera,* which are frugivorous in the larval stage; for example, zingerone is typically found in orchid blossoms (Tan and Nishida, 2000; 2007). Males of individual *Bactrocera* species commonly respond to only one of these chemicals, and the response is so strong that these phytochemicals, or their synthetic analogues, are used as male lures (this terminology used hereafter) in pest management (Metcalf,

74 1990). The two most frequently used are ME and 4-(4-acetoxyphenyl)-2-butanone
75 (cuelure, a raspberry-ketone analogue) (Drew and Hooper, 1981).

76 In utilizing chemical lures, male flies locate a lure source through upwind
77 anemotaxis (Hee and Tan, 1998; Meats and Hartland, 1999), feed on the source, and
78 transport the ingested lures via the haemolymph to the rectal gland (Hee and Tan,
79 2006; Wee and Tan, 2007), which is the site of pheromone synthesis in *Bactrocera*
80 genus (Hee and Tan, 2005). The lures can either be transported to the rectal gland in
81 an unaltered state (e.g., raspberry ketone, Tan and Nishida, 1995) or are converted
82 soon after digestion, and their conversion products are subsequently transported and
83 stored in the rectal gland (e.g., ME which is converted to (*E*)-coniferyl alcohol, Hee
84 and Tan, 2004). It is thought these chemicals are then used within the glands for
85 pheromone synthesis, with rectal gland extracts from lure-fed flies reflecting the
86 presence of the additional chemicals (Hee and Tan, 1998; Tan and Nishida, 1998). In
87 wind-tunnel tests females responded more strongly to odours from lure-fed males than
88 lure-unfed males (Hee and Tan, 1998; Wee et al., 2007) or to point sources of these
89 new pheromone components (Khoo et al., 2000). From such studies, it is assumed the
90 altered pheromones are responsible for the enhanced mating success seen in lure-fed
91 males of several *Bactrocera* species (Shelly, 2010).

92 Queensland fruit fly, *Bactrocera tryoni* (Froggatt), Australia's most
93 pestiferous fruit fly, responds strongly to cuelure/raspberry-ketone (Meats and
94 Hartland, 1999) and very weakly to zingerone (Fay, 2012). The male fly produces a
95 sex pheromone that is considered to be involved in close range female excitation
96 rather than long distance attraction (Fletcher, 1968; Bellas and Fletcher, 1979). The
97 pheromone constituents recovered from the rectal gland of lure-unfed flies are *N*-(3-
98 methylbutylpropanamide), *N*-(3-methylbutylacetamide), *N*-3-methylbutyl-2-

99 methylpropanamide, *N*-2-methylbutylpropanamide, *N*-2-methylbutylacetamide and *N*-
100 2-methylbutyl-2-methylpropanamide (Bellas and Fletcher, 1979). *Bactrocera tryoni*
101 males begin to accumulate ingested cuelure in the rectal gland as raspberry ketone
102 within six hours of feeding, continuing for at least the next 24 hours (Tan and Nishida,
103 1995).

104 As part of a larger investigation of the physiological effects of lure feeding on
105 *B. tryoni*, we found that male consumption of either cuelure and zingerone, not only
106 enhances male mating success, but also affects the egg production and longevity of
107 females with which they mate (Kumaran et al., 2013) and enhances their sons' ability
108 to locate cuelure and zingerone sources (Kumaran and Clarke, 2014). These female
109 and offspring effects have not been previously reported for any *Bactrocera* species.
110 Given that the lures seem to be affecting *B. tryoni* physiology beyond that simply
111 associated with enhanced mating success, we considered it pertinent to revisit the
112 work of Tan and Nishida (1995), who studied cuelure and raspberry ketone
113 accumulation in the rectal glands of *B. tryoni*. Specifically, we wished first to
114 determine whether we could replicate their results for cuelure (which is accumulated
115 in the rectal gland as raspberry ketone) and then to assess whether zingerone is
116 processed by the fly in the same way. We also examined the headspace emission
117 from sexually calling male flies to evaluate whether the chemicals within the rectal
118 gland were those released, something only rarely done in this field (but see Wee et al.,
119 2007) and not previously for a cuelure-responsive species. In addition, to determine if
120 their release is exclusively associated with male pheromone calling, we investigated if
121 cuelure and zingerone (or any related products) were excreted at midday as metabolic
122 by-products for some other physiological purpose rather than only as pheromone
123 volatiles at dusk during calling. Finally, we investigated whether the accumulation of

124 plant compounds increased female attraction, which is considered a mechanism for
125 the enhanced mating success of lure-fed male *Bactrocera*. In summary, our overall
126 aim was to determine if cuelure and zingerone were being processed in a way
127 consistent with their being part of the male sex pheromone (as for other *Bactrocera*),
128 and if lures were used as part of pheromone calling, we studied whether such an
129 altered olfactory signal elicits strong female preference.

130 **2. Materials and methods**

131 *2.1. Insect source*

132 *Bactrocera tryoni* were obtained from an annually refreshed culture
133 maintained by the [Queensland Government] Department of Agriculture, Fisheries
134 and Forestry, Brisbane. Flies were maintained at 27 °C and 70% RH in a room
135 illuminated with fluorescent tubes between 07:00 and 16:00 h and with natural light
136 for the rest of the day. Flies were held in screen cages (90 × 60 × 60 cm) and provided
137 with protein hydrolysate, sugar and water *ad libitum* following the procedures of
138 Heather and Corcoran (1985). Females and males were separated within three days of
139 emergence from pupae before attaining sexual maturity and unmated sexually mature
140 (14-17 day old) flies were used for all experiments.

141 *2.2. Feeding of male lures*

142 Male flies were provided with 1.5 mL of cuelure (International pheromone
143 systems Ltd, >95% purity) or zingerone (10 µg diluted in 1 µL of 95% ethanol) (=
144 vanillyl acetone; Sigma-Aldrich, >96% purity) on a cotton wick placed on an inverted
145 Petri dish for 2h between 08:00 and 10:00 h. The lure dosage used was identified as
146 optimum concentration to elicit a feeding response based on preliminary studies. Flies

147 fed on the lures by licking the cotton wick and presumably imbibing the chemicals.
148 Flies observed to feed on the lure were removed and maintained in new cages and
149 provided with food and water *ad libitum* until used in further studies.

150 *2.3. Rectal gland excision and compound extraction*

151 The rectal gland is an extension of the hindgut used for water absorption in
152 other insects (Wigglesworth, 1932). To dissect out the gland, flies were firmly held
153 ventral side up, and the aedeagus gently pulled out using forceps until the rectal gland
154 was completely revealed. The hind gut was cut using fine scissors, taking care not to
155 lose the gland contents, and the glands were placed immediately in a 2 mL screw-top
156 vial containing 0.5 mL of absolute ethanol. The gland and solvent were sonicated to
157 enhance greater extraction, and 1 μL of the resulting extract was analysed with a gas
158 chromatograph (GC) (Agilent 6890 Series) coupled to a mass spectrometer (MS)
159 (Agilent 5975) (see below for GC-MS conditions). Glands were excised 3 h, 6 h, 1 d
160 and 3 d after ingestion of male lures. For any given period, extracts from four glands
161 were used for injection, because compounds in one gland were determined by
162 preliminary studies to be below the detectable limit by GC-MS. Hence the
163 concentration presented is for four males. The study was performed using two cohorts
164 of flies, and the pooled mean of two cohorts was used to compare lure-fed and lure-
165 unfed groups.

166 *2.4. Trapping volatiles using thermal desorption tubes*

167 To analyse volatiles released by calling males, 14-day old flies were fed with
168 cuelure and zingerone during the morning. Two hours before dusk (=mating window
169 period) on the same day, 20 cuelure-fed, zingerone-fed or lure-unfed males were
170 released separately into sealed 250 mL glass conical flasks with inlet and outlet tubes.

171 Females were kept in a separate flask, in case males needed the visual presence of
172 females to initiate calling (= wing fanning and pheromone release). When first calling
173 was observed, the flask was connected to a pump, and laboratory air (purified by
174 passing through a charcoal filter) was pulled over the males at the rate of 250 mL/min.
175 Volatile compounds were trapped on a thermal desorption tube (Markes) packed with
176 Tenax TA 35/60, Carbograph 1TD 40/60 (344.6 ± 0.748 mg) attached to the outlet.
177 After collecting volatiles, tubes were thermally desorbed (Markes, TD-100) and
178 injected into the GC-MS for analysis (see below for GC-MS conditions). Preliminary
179 work determined that a single day's sampling resulted in concentration below the
180 detection limit of the instrument, so volatiles were collected for 2 h on three
181 consecutive days using the same tube. That is, the concentration of pheromones
182 presented is the cumulative amount produced in three consecutive days by three new
183 groups of 20 males. Collection of headspace volatiles was made from two batches of
184 flies and data were pooled to obtain a grand mean for comparison.

185 *2.5. Temporal and diurnal difference in pheromone volatiles*

186 This study was performed to determine if the ingested compounds are
187 transformed into other compounds over time after 0 d, 1 d or 3 d of lure feeding. For
188 this, 14-day old virgin males were provided with either 1.5 mL of cuelure or
189 zingerone (10 µg/µL of 95% ethanol) and 20 fed males from each group (cuelure or
190 zingerone) were placed separately into a 250 mL glass conical flask 2 h before dusk.
191 After two hours of calling, the droplets of excretions found on the walls of the conical
192 flask (presumed to be male pheromone volatiles) were dissolved in 2 mL of
193 dichloromethane (Sigma, HPLC grade) and transferred into 5 mL glass vials. The
194 samples were sonicated and 1 µL aliquots were analysed by GC-MS (see below for

195 GC-MS conditions). Collections were made at 0, 1 and 3 days after lure exposure with
196 one cohort of flies.

197 To determine if the release of ingested compounds is exclusively associated
198 with male calling and not excreted as metabolic by-products, the same setup used for
199 temporal difference was used but any excretions were collected for 2 h at midday
200 between 12:00 and 14:00 h. The samples were sonicated and 1 µL aliquots were
201 analysed by GC-MS (see below for GC-MS conditions). Collections were made at 0
202 and 3 days after exposure with one cohort of flies.

203 *2.6. Instrument and method profile*

204 Samples were analyzed with a gas chromatograph (GC) (Agilent 6890 Series)
205 coupled to a mass spectrometer (MS) (Agilent 5975) and fitted with a silica capillary
206 column (Agilent, model HP5-MS, 30 m×250 µm ID × 0.25 µm film thickness). GC
207 conditions for acquiring data were - inlet temperature: 250 °C, carrier gas: helium at
208 51 $cm.s^{-1}$, split ratio 13:1, transfer-line temperature: 280 °C, initial temperature: 40
209 °C, initial time: 2 min, rate: 10 $°C.min^{-1}$, final temperature: 260 °C, final time: 6 min.
210 The MS was held at 280 °C in the ion source and the scan rate kept was 4.45 scans per
211 second.

212 *2.7. Chemical analysis*

213 Tentative identities were assigned to peaks with respect to the National
214 Institute of Standards and Technology (NIST) mass spectral library. Mass spectra of
215 peaks from different samples with the same retention time were compared to ensure
216 that the compounds were indeed the same. Retention time and retention index of
217 tentative identities are presented in Table S1.

218 To compare the proportion of one compound with another compound in the
219 same sample, relative peak area ([peak area of the compound/total peak area the
220 sample] × 100) was calculated. To compare the same compound between two
221 samples, for example, concentration of the same compound in lure-fed and lure-unfed
222 flies, comparative peak area ([peak area of compound in one sample/ mean of total
223 peak area of two samples] × 100) was measured.

224 *2.8. Female response to rectal glands and calling males*

225 To determine if the male pheromone is more attractive after lure feeding,
226 attraction of females to rectal glands and live calling males were studied in the
227 laboratory. To test female response to gland contents, males were fed on cuelure or
228 zingerone between 08:00 and 10:00 h, and four rectal glands each from either cuelure-
229 fed, zingerone-fed or lure-unfed males were excised at dusk and placed directly on an
230 inverted glass Perti dish. The Petri dish was placed inside the experimental arena (a
231 Perspex cage, 30 × 20 × 20 cm high) which contained 15 virgin, sexually mature
232 females released 2-3 h before dusk. Ten cages were run for each treatment (cuelure,
233 zingerone and unfed) under no-choice arenas. During the mating period, records were
234 made on the number of females responding to the pheromone source continuously
235 from 17:00 to 18:30 h. Response was assumed to occur when females perched on
236 Petri dish and dragged their ovipositor. Responding females were aspirated out and
237 time of response was noted. Response latency was calculated as time taken to respond
238 to lure source after onset of the observation period (17:00 h).

239 For testing female response to calling males, instead of extracted rectal glands,
240 10 live cuelure-fed, zingerone-fed, or lure-unfed males were placed in a 250 mL glass
241 beaker closed with paraffin film (Pechiney Plastic Packaging Inc.). Pinholes were then

242 made in the paraffin film to facilitate diffusion of pheromone released by calling
243 males. The beaker with its males was placed inside the experimental cage during the
244 mating period (between 17:00 and 18:30 h), and observations identical to that of rectal
245 gland attraction study were made. Response was assumed to occur when females
246 landed on the paraffin film and dragged their ovipositor. Six replicates, each with 15
247 virgin females, were run per treatment under no-choice arenas.

248 The data on female response were subjected to one-way ANOVA using R-
249 3.0.2 software and significance tested using Tukey's HSD at $\alpha = 0.05$. Appropriate
250 transformation of data was made when violation of assumptions was detected, and the
251 data were back transformed to present in figures.

252 **3. Results**

253 *3.1. Rectal gland compounds*

254 Males fed on cuelure or zingerone accumulated the ingested compounds in
255 their rectal glands in their unaltered state or as analogues (Fig. 1). As previously
256 reported, cuelure-fed males stored ingested cuelure predominantly as raspberry ketone
257 (the hydroxyl form of cuelure), but in addition we also recorded a trace quantity of
258 cuelure stored as 4-(4-hydroxyphenyl)-2-butanol. Zingerone ingested by males was
259 stored predominantly in the original form, with some also being converted to
260 raspberry ketone and β-(4-hydroxy-3-methoxyphenyl)-propionic acid. Among the
261 endogenous compounds, *N*-hexylpropanamide was detected in large quantities in all
262 three treatment conditions (Fig. 1 & Table 1). The chemical structure of the
263 endogenous and the exogenous compounds are presented in Fig. S1.

264 *3.2. Bio-transformation in rectal glands*

265 Ingested compounds accumulated as early as 3 h after feeding and were not
266 further transformed 6 h, 1d or 3d post lure feeding, i.e., the blend of ingested and
267 endogenous compounds extracted from the rectal glands did not change over time
268 (Table 1). There was no consistent pattern of increase or decrease in abundance of
269 ingested and endogenous compounds over time; however, the endeogenous
270 pheromone compounds *N*-(3-methylbutylacetamide), *N*-hexylpropanamide and *N*-
271 propylbutyramide] showed increased abundance one day after exposure in the
272 cuelure-fed condition and at 6 h after exposure in the zingerone-fed conditions.

273 *3.3. Pheromonal compounds*

274 Ingested compounds or their analogues were released as volatiles during
275 courtship by lure-fed males (Fig. 2). Along with the endogenous compounds, cuelure-
276 fed males released raspberry ketone, and zingerone-fed males released zingerone,
277 raspberry ketone and 3-hydroxy-2-butanone (Table 2). The endogenous rectal gland
278 extracts *N*-(3-methylbutylacetamide) and *N*- hexylpropanamide were detected as
279 volatiles in the headspace of calling males. However, instead of *N*- propylbutyramide
280 (as detected in rectal gland extracts), the headspace contained 2-hydroxypropanamide,
281 and in zingerone head space volatiles, 3-hydroxy-2-butanone was detected instead of
282 β-(4-hydroxy-3-methoxyphenyl)-propionic acid. Additionally, various propanoic acid
283 derivatives were detected in volatiles released by lure-fed and lure-unfed males, as a
284 result a total of three endogenous chemicals were detected in the rectal gland extract
285 (Table 1), while seven endogenous chemicals were detected in head space (Table 2).

286 *3.4. Temporal and diurnal differences in pheromone volatiles*

287 The composition of the pheromonal blend released did not vary over time for
288 any of the treatments (Table 3). With respect to the abundance of endogenous

289 compounds, *N*-(3-methylbutylacetamide) and *N*-hexylpropanamide decreased from 0
290 d to 3 d post feeding both in cuelure-fed and zingerone-fed males, whereas 2-hydroxy
291 propanamide showed no distinct trend.

292 At midday, 0 and 3 d post lure feeding, none of the endogenous or exogenous
293 pheromonal compounds detected during dusk were found to be released in the
294 secretions of *B. tryoni* males. There were no compounds detected except contaminants
295 (Fig. S2).

296 *3.5. Female response to rectal gland contents and calling males*

297 There was a significant difference in female response to rectal glands of
298 cuelure-fed, zingerone-fed and lure-unfed males ($F_{2,\ 29}$ = 40.62; p <0.001). A
299 significantly greater proportion of females responded to rectal glands of cuelure-fed
300 males than to the glands of zingerone-fed or unfed males, which were not different to
301 each other (Fig. 3a).

302 Female response to the pheromone of calling males also differed significantly
303 among treatments ($F_{2,\ 17}$ = 9.35; p = 0.002). A significantly greater proportion of
304 females responded to cuelure-fed calling males than to unfed males, with the response
305 to zingerone-fed males intermediate between the two (Fig. 3b).

306 There was no significant difference in response latency among the three
307 treatments (rectal glands: $F_{2,\ 142}$ = 1.804, p = 0.168; calling males: $F_{2,\ 112}$ = 1.532, p =
308 0.589). Mean response latency to rectal glands of cuelure-fed, zingerone-fed and
309 unfed males was 59.72 ± 16.73, 57.86 ± 14.74 and 61.69 ± 14.81 min, respectively.
310 Mean response latency to calling males were 63.57 ± 7.26, 63.85 ± 6.04 and 66.29 ±
311 3.00 min, respectively, for cuelure-fed, zingerone-fed and lure-unfed males.

312 **4. Discussion**

313 *4.1. Summary of results*

314 Male *B. tryoni* fed with cuelure or zingerone stored the ingested compounds in
315 their rectal glands with minimal or no chemical transformation. As previously
316 reported (Tan and Nishida, 1995), cuelure-fed males stored cuelure in its hydrolysed
317 form of raspberry ketone, and accumulation of the chemicals in the rectal glands
318 occurred as early as 3h after lure feeding. A minute quantity of cuelure was also
319 converted to 4-(4-hydroxyphenyl)-2-butanol, a chemical also found in *Bulbophyllum*
320 *apertum* Schlechter flowers, which are a natural source of zingerone that attract
321 *Bactrocera* fruit flies in nature (Tan and Nishida, 2005). When zingerone was
322 ingested by flies it was stored in a largely unaltered state, but there was some
323 transformation to raspberry ketone and β-(4-hydroxy-3-methoxyphenyl)-propionic
324 acid. A discussion of these transformations is developed more fully below.

325 The exogenously derived rectal gland compounds were released along with
326 endogenously derived chemicals during courtship. The ingested compounds were
327 released only during dusk and not at midday, confirming their tight association with
328 male calling and pheromone release. The blend of released chemicals was identical
329 between lure-fed and control flies except for the ingested lures and their derived
330 products. With respect to the post-feeding biotransformation of lures, the structure of
331 the pheromone compounds did not change over time either for material in the rectal
332 glands or when released as volatiles during courtship.

333 Female response was greater to the extracted rectal glands of cuelure-fed
334 males and calling males fed with cuelure than to control males, similar to the results
335 for methyl eugenol fed *B. papayae* and *B. carambolae* (Hee and Tan, 1998; Khoo et

336 al., 2000; Wee et al., 2007). In contrast, zingerone in the rectal gland extract or
337 pheromone did not statistically improve the response rate of females over controls.
338 These studies confirm that the addition of phytochemical lures can make pheromone
339 blends more attractive to females in *B. tryoni*, but the response is lure specific.

340 *4.2. Suspected chemical conversions*

341 Within the glands of zingerone-fed flies we detected, in addition to zingerone,
342 small but consistent amounts of raspberry ketone and β-(4-hydroxy-3-
343 methoxyphenyl)-propionic acid. The conversion of zingerone to these products
344 requires at least a two-step enzymatic process involving demethylation and
345 dehydroxylation to remove the methoxy moiety of zingerone (for chemical formulae
346 refer Fig. S1); this processing complexity is the suspected reason why no raspberry
347 ketone was detected in the rectal gland of zingerone-fed *B. cucurbitae* (Coquillett)
348 (K.H. Tan pers. comm.). We have confidence, however, that our results are real. The
349 MS profile of both compounds appropriately matched the MS library (Fig. S3), and
350 we have no *a priori* reason to suspect chemical mismatches. The possibility of sample
351 contamination also needs to be considered, but we think this very unlikely. These
352 chemicals were detected in consistent quantities in all eight independent samples
353 involving zingerone-fed flies in our study but were not detected in any control or
354 midday samples, while β-(4-hydroxy-3-methoxyphenyl)-propionic acid was not
355 detected in any cuelure-fed males. We thus consider that these results, while
356 chemically unexpected, are real.

357 *4.3.* Bactrocera tryoni *pheromone volatiles*

358 The endogenously synthesised spiroacetals *N*-(3-methylbutyl)propanamide, *N*-
359 (3-methylbutyl)acetamide, *N*-(3-methylbutyl)-2-methylpropanamide, *N*-2-

360 methylbutylpropanamide, *N*-2-methylbutylacetamide and *N*-2-methylbutyl-2-
361 methylpropanamide have been previously reported as *B. tryoni* pheromone volatiles
362 after extraction from the rectal glands (Bellas and Fletcher, 1979). The first three
363 (only) of these chemicals were also detected by Tan and Nishida (1995), and it is
364 likely that they are the major pheromone constituents, while the other three chemicals
365 reported by Bellas and Fletcher are possibly reduced forms. In our study we detected
366 *N*-hexylpropanamide, *N*-(3-methylbutyl)acetamide and *N*-propylbutyramide (in
367 decreasing order of abundance) in the rectal glands of both lure-fed and unfed males
368 and, while two out of three of these chemicals are different to those previously
369 reported, they are chemically very close, and we cannot distinguish if the differences
370 are biological or due to processing and profile matching differences. Most
371 importantly, Fletcher and Kitching (1995) reported *N*-(3-methylbutyl)propanamide as
372 a dominant pheromonal compound, and this appears to have been replaced by the
373 related *N*-hexylpropanamide in our study. Additional to core constitutive chemicals,
374 we report various propanoic acid derivatives in the volatiles released by lure-fed and
375 unfed males (Table 2) that were not reported by Fletcher and Kitching (1995).
376 Spiroacetals such as *N*-(3-methylbutyl)propanamide and *N*-(3-methylbutyl)acetamide
377 have also been found in volatiles of female *B. tryoni* (Booth et al., 2006), and a
378 possible two-way pheromonal communication in the *B. tryoni* mating system needs to
379 be examined.

380 *4.4. Implications for understanding lure utilisation by* B. tryoni

381 Numerous studies have shown that the ingestion of specific phytochemicals or
382 their analogues by the males of various *Bactrocera* species enhances their subsequent
383 mating success (Tan and Nishida, 1996, 2000; 2005; Khoo and Tan, 2000; Shelly and
384 Villalobos, 1995; Shelly, 2000a, b), and we have also shown this for *B. tryoni*

385 (Kumaran et al., 2013). The mechanism of mating success is presumed to be due to
386 the alteration of the pheromone released by lure-fed males or enhanced calling,
387 following accumulation of ingested compounds within the pheromone glands (Nishida
388 et al., 1988; Shelly and Dewire, 1994; Tan and Nishida, 1995; Hee and Tan, 2004;
389 2005; 2006; Tan et al., 2011). For *B. tryoni*, we demonstrate that cuelure and
390 zingerone are being incorporated into the male pheromone and are released only
391 during courtship interactions, a result in agreement with these earlier studies.

392 The female response studies showed increased attraction of females to rectal
393 glands of cuelure-fed males and cuelure-fed calling males, a result similar to other
394 species (Hee and Tan, 1998; Khoo et al., 2000; Wee et al., 2007) and a likely reason
395 for the enhanced mating success of cuelure-fed *B. tryoni* reported by Kumaran et al.
396 (2013). Raspberry ketone attracts mature virgin female *B. tryoni* (Fitt, 1981), so its
397 presence in a male pheromone blend may well be beneficial to males. However, in the
398 current study the inclusion of zingerone did not significantly increase female
399 attraction over control treatments, yet zingerone feeding also significantly increases
400 male mating success in *B. tryoni* (Kumaran et al., 2013). Additionally, Kumaran et al.
401 found that both cuelure and zingerone feeding decreased mating latency time in mate
402 choice arenas, but in the current study when rectal glands only were exposed to
403 females, or when calling males were constrained in glass arenas, time until female
404 response did not differ between lure treatments and control. This suggests that the
405 decreased mating latency following lure feeding reported by Kumaran et al. was
406 driven by an altered male behaviour and not by female response to the altered male
407 pheromone.

408 The results of mating latency in zingerone-fed condition suggest that, for *B.*
409 *tryoni* at least, a pheromonal mechanism is not the sole explanation of male mating

410 advantage following lure feeding. Both raspberry ketone and zingerone are well
411 recognised metabolism enhancers in a wide range of organisms (Venkatramalingam et
412 al., 2007; Park, 2010; Chang et al., 2012) and, if this effect also occurs in fruit flies,
413 then it is possible that the chemicals may play multiple, and possibly synergistic roles,
414 in the mating system. For example, in *B. tryoni,* cuelure consumption may not only
415 make the male pheromone more attractive, but it may also make the males more
416 physically active and so more competitive. Zingerone consumption may not directly
417 enhance the pheromone blend, but it may make the males more active and so they
418 may release more of the endogenous pheromone chemicals as enhanced male calling
419 evidenced in *B. dorsalis* after methyl eugenol feeding (Shelly and Dewire, 1994), or
420 they may simply be better able to physically compete against lure-unfed males.

421 *4.5. Conclusion*

422 This study confirms that ingested phytochemical lures modify the composition
423 of *B. tryoni* pheromone volatiles and, for cuelure but not zingerone, this modified
424 pheromone subsequently attracts more females. While the enhanced mating success
425 documented in cuelure-fed *B. tryoni* males may be explained by the altered
426 pheromone, a pheromone hypothesis does not explained enhanced mating success in
427 zingerone-fed *B. tryoni* males, and additional explanations are needed. As more
428 *Bactrocera* species are tested, and different lures are tested on the same species, it
429 becomes increasingly clear that, beyond the recognition that the male lures are
430 intimately associated with the mating systems of *Bactrocera* fruit flies, their role for
431 individual species cannot be generalised.

432

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443 **Figure captions**

444 **Fig. 1** Chromatogram showing compounds detected in rectal gland extracts 6 h post-
445 feeding from *Bactrocera tryoni* males fed on (a) cuelure, (b) zingerone and (c) unfed
446 males. [1) *N*-(3-methylbutyl)acetamide; 2) *N*-hexylpropanamide; 3) *N*-
447 propylbutyramide; 4) 4-(4-hydroxyphenyl)-2-butanone (raspberry ketone); 5) 4-(4-
448 hydroxyphenyl)-2-butanol; 6) 1*H*-indole-3-ethanol; 7)) 4-(4-hydroxy-3-methoxy
449 phenyl)-2-butanone (Zingerone); 8) β -(4-hydroxy-3-methoxyphenyl)-propionic acid;
450 9) 4-(4-hydroxy-3-methoxyphenyl-3-buten-2-one); 10) 4-(3-hydroxy-2-
451 methoxyphenyl)-butan-2-one)]

452 **Fig. 2** Stackplot of chromatograms showing volatile compounds detected during
453 courtship in cuelure-fed, zingerone-fed and unfed *Bactrocera tryoni* males. [1) *N*-(3-
454 methylbutyl)acetamide; 2) *N*- hexylpropanamide-3-methylbutanol; 4) 4-(4-
455 hydroxyphenyl)-2-butanone (raspberry Ketone); 7) 4-(4-hydroxy-3-methoxyphenyl)-
456 2-butanone (zingerone); 11) 2-hydroxypropanamide ; 12) 2-methylethyl ester
457 propanoic acid; 13) 2-methylpropanoic acid; 14) 1-methyl undecyl ester propenoic
458 acid]

459 **Fig. 3** Mean percentage (+ SE) of *Bactrocera tryoni* female response to a) male rectal
460 glands (n = 10 per treatment) and b) calling males (n = 6 per treatment) after males
461 were fed with cuelure, zingerone and unfed males. Different letters on adjacent bars
462 indicate significant difference ($P < 0.05$) in female response between treatments

463

464 **Electronic supplementary documents**

465 Fig. S1 Structure of plant secondary metabolites that *Bactrocera tryoni* feed on and
466 the structure of endogenously synthesised volatiles released during courtship

467 Fig. S2 Stackplot of chromatogram showing no evidence of detection of pheromone
468 volatiles during midday from *Bactrocera tryoni* males fed on cuelure, zingerone and
469 unfed males

470 Fig. S3 Mass spectrometry (MS) library matching profile of (a) raspberry ketone and
471 (b) β-(4-hydroxy-3-methoxyphenyl)-propionic acid detected in zingerone-fed males

472 Table S1. Retention time and retention index of tentative compounds identified from
473 pheromone volatiles and rectal gland extracts of male *Bactrocera tryoni*

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