Dufour's gland analysis reveals caste and physiology specific signals in *Bombus impatiens*

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Abstract

Reproductive division of labor in insect societies is regulated through multiple concurrent mechanisms, primarily chemical and behavioral. Here, we examined if the Dufour's gland secretion in the primitively eusocial bumble bee *Bombus impatiens* signals information about caste, social condition, and reproductive status. We chemically analyzed Dufour's gland contents across castes, age groups, social and reproductive conditions, and examined worker behavioral and antennal responses to gland extracts.

We found that workers and queens each possess caste-specific compounds in their Dufour's glands. Queens and gynes differed from workers based on the presence of diterpene compounds which were absent in workers, whereas four esters were exclusive to workers. These esters, as well as the total amounts of hydrocarbons in the gland, provided a separation between castes and also between fertile and sterile workers. Olfactometer bioassays demonstrated attraction of workers to Dufour's gland extracts that did not represent a reproductive conflict, while electroantennogram recordings showed higher overall antennal sensitivity in queenless workers. Our results demonstrate that compounds in the Dufour's gland act as caste- and physiology-specific signals and are used by workers to discriminate between workers of different social and reproductive status.

Introduction

Reproductive division of labor in insect societies is regulated through multiple concurrent mechanisms. These often involve a combination of interactions with brood[1,2], agonistic and policing behaviors among nestmates[3–5], and chemical signaling among individuals in the colony[6–8]. In the latter, chemical signals from the queen can affect worker physiology and behavior by reducing their ovarian activation[9–12] and by marking eggs to indicate their maternity, which prevents their consumption by "policing" workers[13–16]. In workers, chemical signals can indicate dominance and fertility status[17–19], which increases aggressive behavior towards fertile workers[20] or indicate sterility, which appease aggressive individuals[8,21]. Across social insects, these signals are chemically diverse and have multiple glandular origins[22–24].

In female Hymenoptera, one such glandular origin is the Dufour's gland (DG), which is associated with the sting apparatus in females and produces a remarkable diversity of compounds with myriad functions[25,26]. In solitary bees, the DG secretion functions primarily in waterproofing brood cells[27,28], although there is also evidence that it is the source of a sex pheromone in two parasitoids[29], and may serve as a nest-marking pheromone in certain solitary bee species[30,31]. In social bees, analyses of the DG secretion have suggested additional functions in social communication[32], such as signaling of reproductive status and the possible regulation of worker aggressive and foraging behaviors in honey bees and bumble bees[8,21,33,34].

Bumble bees provide an excellent opportunity to study the role of chemical signals in the regulation of reproduction because they exhibit characteristics of both simple and highly eusocial insect societies. In the first part of the colony cycle, reproduction is dominated by a single queen, with workers being functionally sterile. This changes in the second half of the colony cycle, the competition phase, where workers initiate egg laying and produce males in competition with the queen[35]. In *Bombus terrestris,* the DG secretion varies with caste, reproductive state, age, and task[8,34]. In queens, the DG contains aliphatic saturated and unsaturated hydrocarbons, whereas in workers these are accompanied by long chain esters. The reduction of these esters to non-detectable amounts in queenless workers with activated ovaries suggests that they may signal

63 sterility[8]. There is also a negative correlation between the amount of esters a worker possesses
64 and the aggression directed toward her during worker-worker competition over reproduction,
65 suggesting an appeasement effect of these esters, in line with their hypothesized function as
66 sterility signals[21]. It is unknown if these signals are widespread in *Bombus*, or represents a unique
67 chemical signaling mechanism in *B. terrestris*. The ester amount in *B. terrestris* also increases with
68 age in a similar fashion as the increase in worker ovarian activation and is therefore an important
69 confounding factor to evaluate when examining the glandular compounds and their relation to
70 social and reproductive status[8]. In this study, we leverage our understanding of *B. terrestris*
71 chemical ecology in order to glean new insights into potential shared or elaborated mechanisms
72 mediating social organization across bumble bee species.
73
74 To examine the role of the gland from a signal production standpoint and discover if *B. impatiens*
75 females produce a social signal in the DG linked to their fertility status, we examined the chemical
76 composition of the secretion as a function of caste, age, social, and reproductive status. To
77 examine the signaling role of the gland from a sensory standpoint, we tested the behavioral and
78 antennal responses of workers, either queenless or queenright, to the DG secretion of either
79 queenless or queenright workers. The DG may carry multiple social signals which are caste or
80 social status dependent. Depending on what these roles are, DG compounds may induce
81 attraction or avoidance to facilitate cooperation or competition. Therefore, we hypothesize that
82 if workers produce a cooperative signal (e.g., sterile workers producing esters, like in *B.*
83 *terrestris*), a DG extract of these workers will elicit higher attraction by cooperative workers in
84 comparison with a control extract, and if workers produce a fertility signal, a DG extract of these
85 workers will elicit avoidance in competing workers as compared to control. Additionally, we
86 hypothesize that the secretion from workers carrying a cooperative or competitive signal will
87 elicit stronger antennal responses in the workers receiving these signals, reflecting increased
88 sensitivity towards compounds that may impact their fitness.
89
90 **Materials and Methods**
91 *Bee colonies and sample preparation for chemical analysis*

92 Bees were collected from *B. impatiens* colonies obtained from Koppert Biological Systems
93 (Howell, Michigan, USA) or Biobest Canada Ltd. (Leamington, ONT). Upon arrival to the
94 laboratory, colonies were approximately 2-4 weeks old (based on first worker emergence), with
95 less than 30 workers each, a queen, and all stages of brood. Colonies were maintained in nest-
96 boxes at a constant temperature of 28–30°C and 60% relative humidity, constant darkness and
97 supplied *ad libitum* with a 60% sugar solution and honeybee-collected pollen. These colonies
98 were used as sources of newly-emerged workers (<24 h old) that were sampled prior to sexual
99 production phase (i.e., gynes and males). Newly-emerged workers were collected from 7 colonies
100 (see Table S1 for sample size) assigned to one of three treatments: queenright (QR, n=70),
101 queenless (QL, n=70), and queenless and brood-less (QLBL, n=70) workers. QR workers were
102 individually labeled on the day of emergence and remained in their natal colony in the presence
103 of the queen until the day of collection while QL and QLBL workers were housed in a plastic cage
104 (11 cm diameter × 7 cm height) in groups of 3-6 workers without a queen. Queenless groups of
105 workers typically lay eggs within 6-8 days[36], and the presence of brood may also affect worker
106 reproduction[2]. Therefore, in the QL groups, eggs laid by workers remained in the cages, whereas
107 in the QLBL groups, eggs laid by workers were removed daily. QR workers were collected from
108 young queenright colonies showing no signs of competition over reproduction between the
109 queen and the workers. Workers ranging in age from one to fourteen days old (5/age group)
110 were collected from all treatments. All workers were collected and stored at −80° C until
111 dissection.
112 Active mated, egg-laying queens (hereafter, "queens"; n=20) were obtained from full-sized
113 colonies with >100 workers. These queens were actively producing female workers prior to
114 sampling. Unmated queens (hereafter "gynes"; n=20) were collected from 3 colonies (see Table
115 S1 for details). They were separated from their natal colonies upon emergence to prevent mating,
116 housed in small cages in groups of 3-5 gynes and sampled at 4 age points: 3, 6, 10, and 14 days
117 after emergence (5/age group).
118 Olfactometer and electrophysiology bioassays were conducted using 7-day old QR and QLBL
119 workers exposed to DG extracts of either 7-day old QR or QLBL workers, based on differences
120 observed in the chemical analysis and ovarian activation in these groups. Olfactometer bioassays

121 were conducted using 122 workers in total taken from 11 colonies and electrophysiology
122 bioassays were conducted using 29 workers taken from 3 colonies (see Table S1 for breakdown
123 per treatment).
124
125 Dissection of bees
126 Ovaries were dissected under a stereomicroscope in distilled water, and the largest three
127 terminal oocytes across both ovaries (at least one from each ovary) were measured with an
128 eyepiece micrometer. The mean of these three oocyte measurements was used as an indicator
129 of ovary activation for each bee. Mean terminal oocytes (hereafter, "oocyte size") was used as a
130 continuous variable in Figures 2a and 4a. In order to perform the LDA analysis in Fig. 3 we had to
131 categorize bee oocyte size as LDA cannot be performed using continuous variables. We therefore
132 categorized workers into three groups based on their terminal oocyte size: inactive (<0.5 mm),
133 intermediate (0.5 – 2 mm), and active (>2 mm).
134 DGs from each individual were dissected and immediately immersed in hexane. Glands were
135 extracted for 48 hours after which each sample was evaporated and reconstituted in 100 µL
136 hexane spiked with an internal standard of 1 µg eicosane (C_{20}).
137
138 Chemical analysis
139 To identify compounds in the DG, extracts were analyzed on two separate mass spectrometers.
140 At UC Riverside, samples were analyzed on a Hewlett-Packard (H-P, now Agilent Technologies,
141 Santa Clara CA, USA) 6890 gas chromatograph (GC) equipped with a DB-17 mid-polarity column
142 (0.25 mm id x 30 m x 0.25 µm film thickness; J&W Scientific, Folsom CA, USA) interfaced to an H-
143 P 5973 mass spectrometer. The temperature program was as follows: 100˚C (0 min. hold) to
144 160˚C at 20˚C/min, then 4˚C/min to 280˚C (hold for 25 min). Injections were made in split-less
145 mode, with injector and transfer line temperatures of 280˚C. At Penn State, samples were
146 analyzed on an Agilent 7890A GC equipped with a HP-5ms column (0.25 mm id x 30 m x 0.25 µm
147 film thickness, Agilent) and interfaced to an Agilent 5975C mass spectrometer. Compounds were
148 tentatively identified based on diagnostic ions in the resulting spectra and retention indices, and

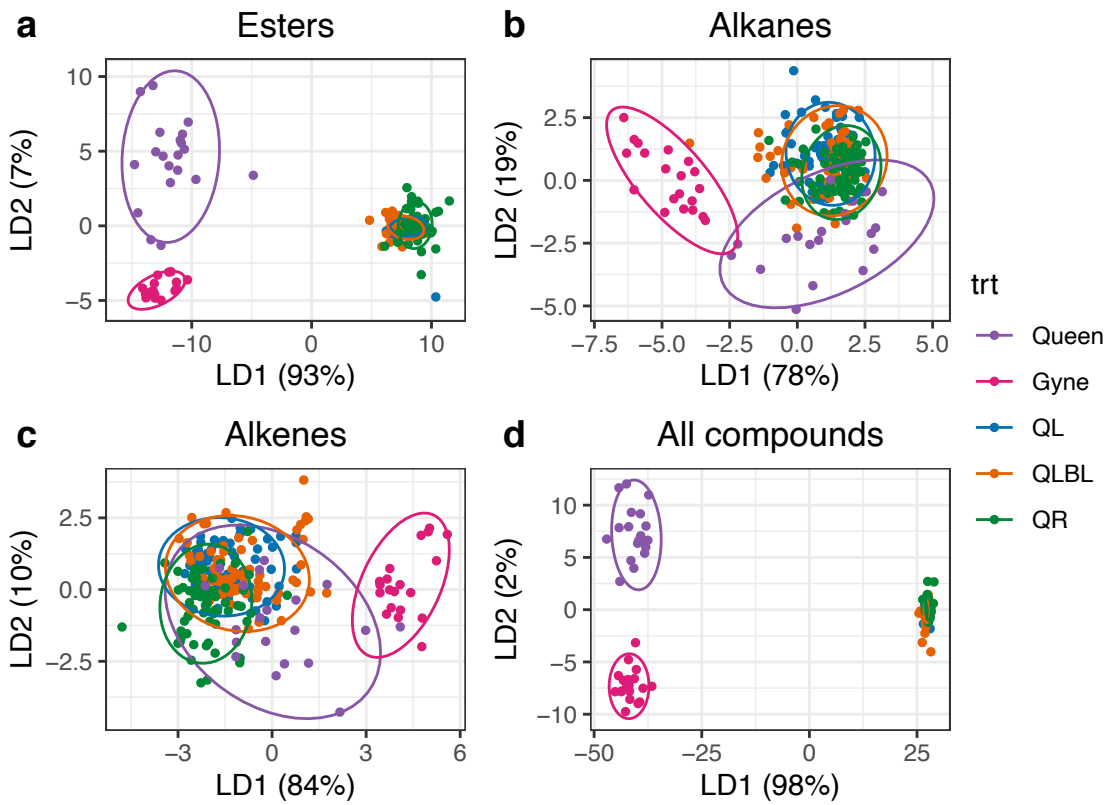
149 where possible, identifications were confirmed by matching retention times and mass spectra
150 with those of authentic standards.
151 Compounds in DG extracts were quantified on a Trace 1310 GC (Thermo Fisher, Waltham, MA
152 USA) equipped with a flame-ionization detector (FID) and a TG-5MS column (0.25 mm id x 30 m
153 x 0.25 µm film thickness; Thermo Fisher). The temperature program was as follows: 60˚C to 120˚C
154 at 15˚C/min followed by 120˚C to 300˚C (5m hold) at 4˚C/min. The injector port and FID were
155 held constant at 250˚C and 320˚C, respectively.
156
157 Olfactometer Bioassays
158 To examine if *B. impatiens* workers perceived the DG secretion by olfaction, and if their response
159 depended on reproductive or social state, bioassays were conducted using two-choice
160 olfactometers as previously described[38]. Olfactometers (see Fig. S1 for illustration) were
161 fashioned from plastic petri dishes (150 × 15 mm) with two equidistant holes (2 cm diameter) in
162 the bottom providing access to small plastic cups that held treatment or control stimuli (QR or
163 QLBL DG extract or control). One gland equivalent of extract (100 µl total volume) was pipetted
164 onto glass coverslips and the solvent was allowed to evaporate before being introduced into the
165 cups, which were subsequently affixed to the olfactometer. After lifting the petri dish lid enough
166 to allow entry, workers were placed in the center of the olfactometer and given 60 min to make
167 a first choice, which was recorded. Bees did not have the ability to touch the glass slides unless
168 they fully entered the cup, and were prevented from returning to the main arena after a choice
169 was made via a 3 cm section of plastic straw glued to each hole. Bioassays were conducted under
170 a red light and olfactometers were washed with soap and water and allowed to air dry before
171 being reused. Cups containing test stimuli, extracts and bees were only used once. All behavioral
172 assays were conducted in the rearing chamber between noon to 4 PM. Seven-day old QR or QLBL
173 workers were tested for their preference between 2 choices in three bioassays: QR DG extract
174 vs. callow DG extract, QLBL DG extract vs. callow DG extract, and QR DG extract vs. QLBL DG
175 extract. Overall, we conducted 122 bioassays (16-25 replicates per treatment group and social
176 condition) which resulted in 71.3% response rate. Bioassays where bees did not respond within
177 the time frame were not included in the analysis. Non-responder bees were not associated with

178 any specific treatment (proportion test, $\chi^2 = 0.16$, $p = 0.69$, 25.9% and 30.9% of QLBL and QR non-
179 responders, respectively). Callow DG extract was used as control as it is undifferentiated into a
180 specific chemical profile, being mostly devoid of potential signal molecules, but went through the
181 same extraction process as 7-day old worker DG. This captured methodological noise that a
182 solvent control would not. In addition, the preference to a solvent control and callow extract
183 were not significantly different when compared directly (binomial exact test, $p = 0.77$, $n = 12$,
184 20% non-responders, data not shown). In all bioassays, extracts were presented to workers as a
185 dose of 1-bee equivalent. Extracts were prepared as described above for the chemical analyses,
186 pooled, and the concentration was adjusted to 1 gland equivalent per 100 µL hexane.
187
188 Electroantennogram recordings
189 Odor cartridges were created using 7-day old worker DG extracts, either QR or QLBL, as follows.
190 1 DG equivalent per 100 µL of hexane (DG extracts were prepared as above) was administered
191 onto strips of Whatman filter paper. After the solvent had evaporated, the filter paper strips were
192 inserted into glass pipettes and sealed with aluminum foil until use. A "blank" control consisting
193 of 100 µL of solvent was allowed to evaporate from the filter paper before being placed and
194 sealed in the cartridge. For the electroantennograms (QR n=13, QLBL n=16), the right antenna
195 was excised and the tip snipped with a fine scissors prior to mounting on a quadroprobe
196 electroantennogram system with Spectra 360 electroconductive gel[39]. A constant airflow of
197 charcoal-purified humidified air was passed across the antenna through a 10 mm glass tube for
198 the duration of each experiment. Each antenna was allowed to reach a steady baseline before
199 starting the experiment, at which point the test odorants were delivered into a steady airstream
200 using the odor cartridges in conjunction with a stimulus flow controller (Syntech, Hilversum,
201 Netherlands). A 40 ml/s air pulse was pushed through the cartridge for 0.05 seconds, effectively
202 delivering 2 ml of volatiles into the air stream and across the antenna. Responses to each stimulus
203 were amplified using a quadroprobe, 10x pre-amplifier (Syntech, Netherlands) and further
204 amplified 10x before being recorded and analyzed on a desktop computer using custom software
205 (as in[40]). Odorants from control, QLBL, or QR extracts were presented to each antenna 30 seconds
206 apart, after the antennal response had returned to baseline activity. Responses to a final blank

207 puff were subtracted from the responses to the test odorants and the corrected values were used
208 in analyses. Each worker antenna was exposed to control, QLBL, and QR extracts, and each odor
209 cartridge was used twice.
210
211 Statistical analysis
212 Statistical analyses were performed using R (version 3.6.1) in RStudio (version 1.2.1335) and SPSS
213 v.21 (IBM) software. To examine differences in the DG secretion between females of differing
214 caste (queen, workers) and social conditions (QR, QL, QLBL worker groups), linear discriminant
215 analyses (LDA) were performed using Aitchinson Z-transformed relative peak areas as predictor
216 variables, and social condition, caste, or age as grouping variables. Only peaks > 0.5% of the total
217 peak area were used. These selected peaks were then re-standardized to 100% and transformed
218 using Aitchinson's formula [$Z_{ij} = \ln[Y_{ij}/g(Y_j)]$[41], to reduce collinearity prior to multivariate
219 analysis[42,43]. For peaks that were not present in all groups, a small nominal value was added as
220 the transformation cannot be calculated with zero values. In some cases, poorly separated
221 clusters of peaks prevented accurate integration, and after mass spectral confirmation that these
222 were of the same compound class (e.g. esters), they were integrated together as a "complex"
223 and used as a single variable.
224 The effects of colony, age, treatment and interactions between them on oocyte size and the
225 amounts of DG compounds were analyzed using SPSS. Neither oocyte size nor absolute amounts
226 of esters, hydrocarbons and total secretion in DG were normally distributed. For this reason, all
227 these parameters underwent a Box Cox transformation prior to being included in the model.
228 Oocyte size was analyzed using Generalized Linear Mixed Models (GLMM) analysis with
229 treatment, age in 2-day intervals and the interaction between them as fixed factors and colony
230 as a random factor. Robust estimation was used to handle violations of model assumptions and
231 Satterthwaite correction was employed to account for small and unequal sample sizes. Absolute
232 amounts of esters, hydrocarbons and total secretion in DG were analyzed using GLMM with
233 treatment, age in 2-day intervals and the interaction between them as fixed factors, oocyte size
234 and its interaction with treatment as covariates, and colony as a random factor. Robust
235 estimation was used to handle violations of model assumptions and Satterthwaite correction was

236 employed to account for small and unequal sample sizes. For all categorical fixed factors a post-
237 hoc contrast analysis with Least Significant Difference (LSD) correction for multiple comparisons
238 was performed. Wilcoxon tests were used to compare oocyte size, ester and hydrocarbon
239 amounts, and ester proportions between QLBL and QR 6 and 7-day old workers. Choice data from
240 olfactometer bioassays were analyzed using exact binomial tests, calculating two-tailed
241 probabilities for a difference in the proportion of choices against the null hypothesis of 50%, or
242 no preference. The average strength of antennal responses from electroantennogram recordings
243 were compared using t-tests when comparing responses based on antennal social condition, and
244 paired t-tests when comparing antennal responses within social condition and between extract
245 type. Significance was evaluated at the $\alpha = 0.05$ level.
246
247 **Results**
248 **Chemistry of *Bombus impatiens* Dufour's gland**
249 DG extracts and the average oocyte size were analyzed in 210 workers (5/age group, ages 1-14
250 days for 3 treatment groups), 20 gynes (5/age group, ages 3, 6, 10, 14 days), and 20 queens (4-
251 15 weeks from the emergence of the first worker). During analysis, we discovered 11 outlier
252 worker samples, which were excluded from further analyses. One QR worker had fully activated
253 ovaries at 8 days of age, which was a clear outlier, and 10 QL workers on days 9 and 10 sharing
254 the same 2 cages and from the same colony had unusually undeveloped ovaries which likely
255 resulted from a shared effect related to rearing conditions (e.g., underfeeding) or maternal
256 colony. Thus, 199 workers were included in subsequent analyses. Overall, GC-FID analysis of *B.*
257 *impatiens* DG extracts in females revealed 98 compounds comprised of aliphatic alkanes (C_{21}-
258 C_{31}), alkenes, wax esters, and diterpenes (Fig. S2, Table S2). Of these, we included in the statistical
259 analysis 28 compounds that were present in the majority of samples and each constitutes at least
260 0.5% of the total secretion.
261
262 **Examining caste specificity in Dufour's gland secretion**
263 The DGs of *B. impatiens* females contain compounds that are both queen-specific and worker-
264 specific, in addition to linear alkanes and alkenes found in both castes (Fig S2). Gynes and queens

265 differed from workers based on the presence of the diterpenes β-springene and isomers, which
266 were absent in workers, and long chain esters that were specific to gynes. Conversely, three
267 dodecyl esters (dodecyl octanoate, dodecyl decanoate, and dodecyl dodecanoate), and
268 hexadecyl decanoate were specific to workers (Fig S2, Table S2). In addition, workers produced
269 large quantities of a fifth ester, dodecyl oleate, which was present only in trace amounts in gynes.
270 In gynes and queens, β-springene accounted for 3.2% ± 0.19 and 1.3% ± 0.33 (mean ± SE, n=20),
271 on average, of the total secretion, respectively. The worker-specific esters comprised, on
272 average, 1.0% ± 0.07, 2.6% ± 0.08, 1.8% ± 0.08, 1.5% ± 0.13, and 5.1% ± 0.28 (mean ± SE, n=199)
273 of the total secretion across workers of all ages and treatments, respectively. Long chain esters
274 found in gynes (tentatively identified as terpene esters 6 and 7) comprised, on average, 1.2% ±
275 0.18 and 2.2% ± 0.26 (mean ± SE, n=20) of the gyne total secretion, while only being detected in
276 trace amounts in workers, and at <1% in DG extracts of queens.
277 Discriminant analyses using either all compounds or specific chemical classes (esters, alkanes,
278 and alkenes) as predictor variables showed that esters enabled differentiation between queens,
279 gynes, and workers with similar explanatory power as the analyses using all compounds (Fig 1).
280 Figure 1 shows plots of the data according to the first two linear discriminant functions based on
281 the compound class used as predictor variables. Of the worker esters, dodecyl decanoate
282 provided the greatest separation between queen and worker castes along LD1 (coefficient of LD1
283 1.71, % variation explained = 93%), while of the gyne esters, terpene esters 6 and 7 accounted
284 for the most variability along LD2 (coefficients of LD2 0.94 and -1.22 respectively, % variation
285 explained = 7%).
286 Figure 1 - Linear discriminant analyses on Aitchison's Z-transformed relative peak areas of
287 Dufour's gland extracts of workers of three social conditions (queenright – QR, queenless - QL,
288 and queenless, brood-less - QLBL), gynes, and queens, with caste/treatment as the grouping
289 variable. Each point is an individual bee. Panels **a-d** show the clustering generated by plotting
290 the first two linear discriminant functions using the esters, alkanes, alkenes, or all compounds.
291



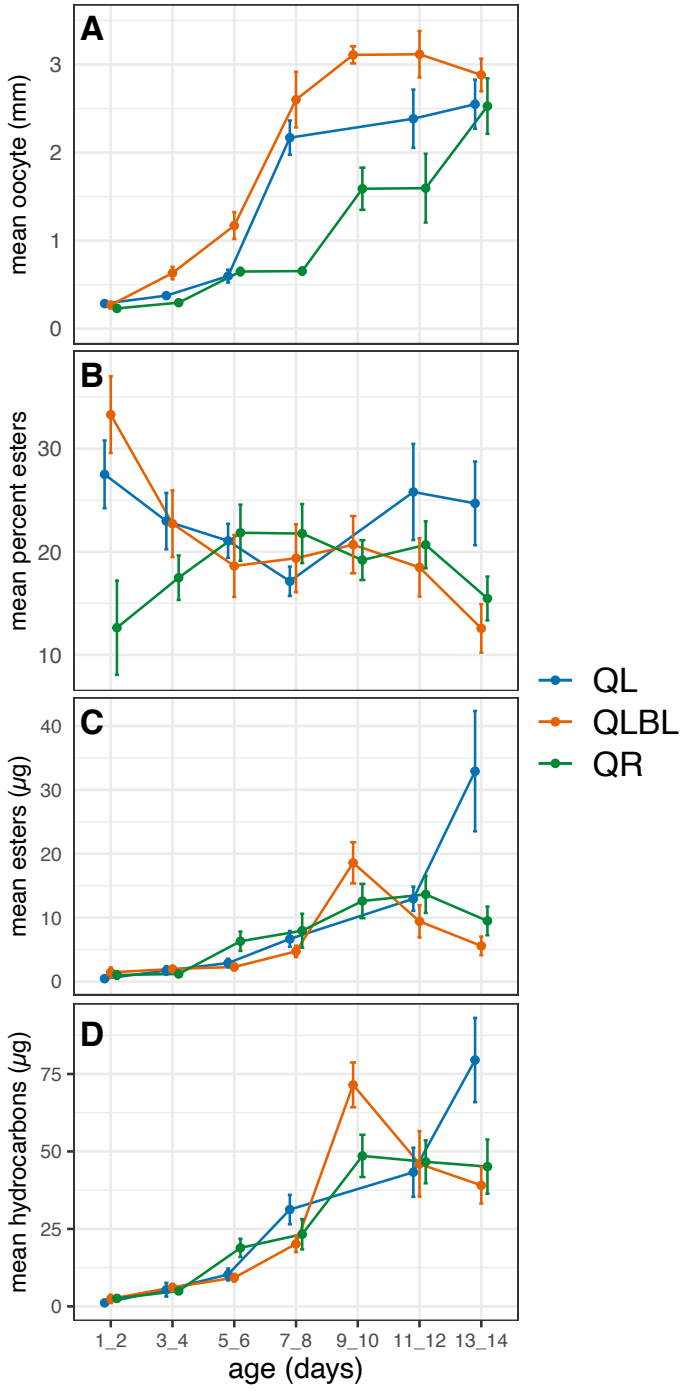
Examining the effect of age, social condition, and reproductive state on worker Dufour's gland secretion

The discriminant analysis data indicate clear separation between queens, gynes, and workers, but produced little to no separation between the worker groups (Fig. 1). However, the large differences between the castes may have masked the more subtle differences between workers. Therefore, we analyzed the three worker groups separately from gynes and queens and tested the effects of worker age, social conditions, and reproductive status on their DG composition.

In Fig. 2 we show data pertaining to the overall changes observed in oocyte size, DG esters (absolute and relative amounts) and hydrocarbon absolute amounts in workers as function of their age and social conditions. In the description below we also provide information on the interactions between these variables, and between further variables not shown in Fig. 2 such as the DG total secretion (which is the sum of esters and hydrocarbons). We show the split of hydrocarbons to alkanes and alkenes in Fig. S3.

Figure 2 – Change in worker reproductive state and Dufour's gland chemistry as a function of age (1-14 days post emergence) and social condition: queenright (QR), queenless (QL), and queenless, brood-less (QLBL) workers. (A) mean terminal oocyte size (mm), (B) mean percent of

total esters, (C) mean µg of total esters, and (D) mean µg of total hydrocarbons. Data are grouped into two-day bins and represent the mean value of 10 workers per age group ± S.E.M.



Oocyte size

Oocyte size was significantly affected by social condition and age with a significant interaction between the two (GLMM, $F_{6,175} = 73.13$, $p < 0.001$ for age, $F_{2,179} = 37.26$, $p < 0.001$ for social

316 condition, $F_{11,175} = 3.44$, $p < 0.0001$ for interaction, Fig. 2A). QLBL workers had larger oocytes than
317 both QL and QR workers (post-hoc LSD pairwise contrast, $p < 0.001$) and QL workers, on average,
318 had slightly larger oocytes than QR workers (post-hoc LSD pairwise contrast, $p = 0.05$). Oocyte
319 size started increasing on days 3-4 and reached a plateau on days 7-8 (post-hoc LSD pairwise
320 contrast, $p < 0.01$ for days 1-2 vs. 3-4, 3-4 vs. 5-6, 5-6 vs. 7-8, $p > 0.05$ for days 7-8 vs. later time
321 points). Post-hoc analysis of the interaction between social condition and age revealed that
322 oocyte size increased with age similarly in QL and QLBL groups (post-hoc LSD pairwise contrast,
323 $p < 0.01$ for days 1-2 vs. 3-4, 3-4 vs. 5-6, 5-6 vs. 7-8, $p > 0.05$ for days 7-8 vs. later time points),
324 while in QR groups the increase started at a later age (5-6 days) (post-hoc LSD pairwise contrast,
325 $p > 0.05$ for days 1-2 vs. days 3-4 and days 9-10 vs. later time points, $p < 0.01$ for other
326 comparisons). QLBL workers developed larger oocytes than those in other groups by days 3-4
327 and the difference persisted at later time points (post-hoc LSD pairwise contrast, $p > 0.05$ at days
328 1-2, $p < 0.01$ for all other comparisons). Based on these differences, we disentangle the effects
329 of age and oocyte size on DG chemistry by including oocyte size as a covariate in all subsequent
330 models.
331
332 Ester proportion
333 The relative proportion of DG esters was significantly affected by social condition and age with
334 significant interaction between the two factors (GLMM, $F_{6,173} = 3.08$, $p = 0.007$ for age, $F_{2,172} =$
335 6.46, $p = 0.002$ for social condition, $F_{11,173} = 2.41$, $p = 0.008$ for age*social condition
336 interaction). Ester proportion covaried with oocyte size ($F_{1,173} = 13.06$, $p < 0.001$) with
337 significant interaction between oocyte size and social condition($F_{2,175} = 7.37$, $p = 0.001$) (Fig.
338 2B). Overall, QR workers had lower proportion of esters than QL and QLBL workers (post-hoc
339 LSD pairwise contrast, $p = 0.001$ for QLBL vs. QR, $p = 0.019$ for QL vs. QR and $p = 0.205$ for QL vs.
340 QLBL), but the differences were mostly driven by the early and late timepoints, and the
341 proportions fluctuate significantly over the examined age groups (post-hoc LSD pairwise
342 contrast, $p < 0.05$ for comparisons of days 1-2 to 7-8 and 9-10 vs. 13-14, $p > 0.05$ for all other
343 comparisons).
344

345 Ester amount

346 The amount of esters in the DG was significantly affected by age but not by social condition,
347 without significant interaction between the two factors, and without covariance with oocyte
348 size or interaction between oocyte size and social condition (GLMM, $F_{6,173} = 5.75$, $p < 0.001$ for
349 age, $F_{2,174} = 1.41$, $p = 0.25$ for social condition, $F_{11,172} = 1.66$, $p = 0.087$ for age*social condition
350 interaction, $F_{1,175} = 0.188$, $p = 0.67$ for oocyte size, $F_{2,174} = 0.01$, $p = 0.99$ for oocyte size*social
351 condition interaction, Fig. 2C). Post hoc analysis revealed that esters amount increased with
352 age up to days 9-10 (post-hoc LSD pairwise contrast, $p < 0.05$ for comparisons of days 1-2 to 7-
353 8, $p > 0.05$ for all later time points).

354

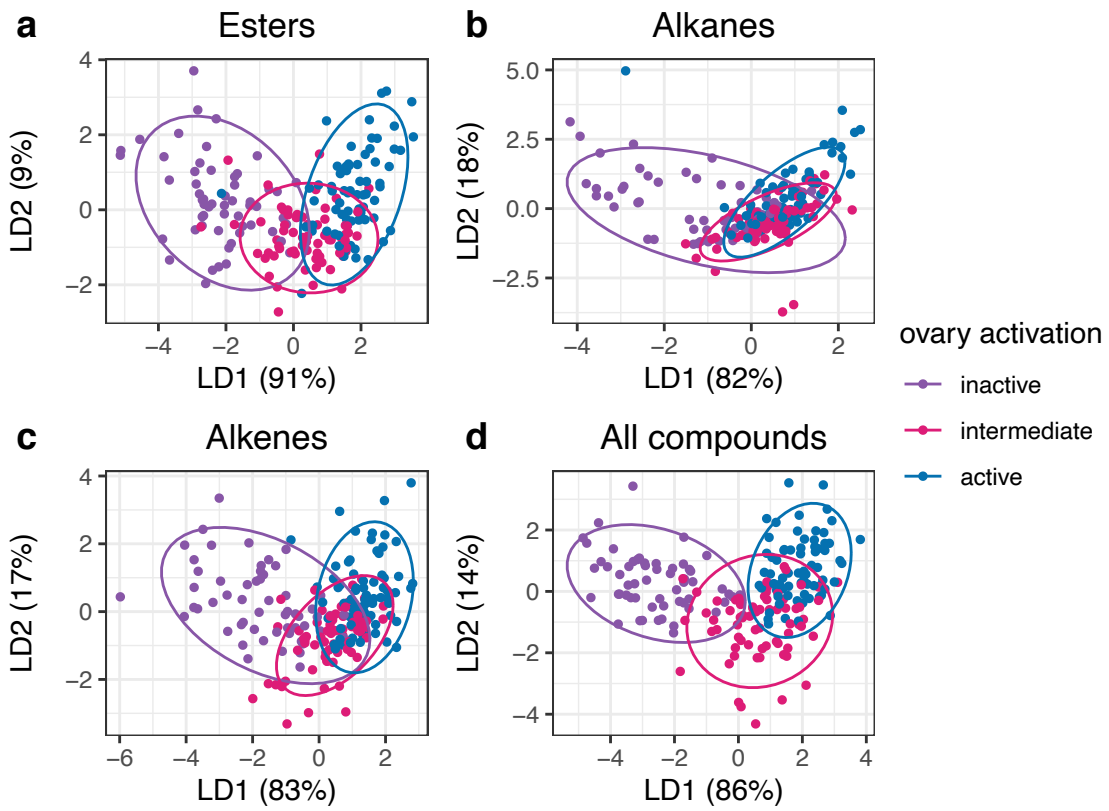
355 Hydrocarbon amount

356 The amount of DG hydrocarbons was significantly affected by social condition and age with
357 significant interaction between the two factors. Hydrocarbons amount covaried significantly
358 with oocyte size with significant interaction between oocyte size and social condition (GLMM,
359 $F_{6,173} = 9.06$, $p < 0.001$ for age, $F_{2,174} = 7.46$, $p = 0.001$ for social condition, $F_{11,173} = 2.45$, $p =$
360 0.007 for age*social condition interaction, $F_{1,175} = 19.23$, $p < 0.001$ for oocyte size, $F_{2,174} = 4.89$,
361 $p = 0.009$ for oocyte size*social condition interaction, Fig. 2D). Overall, QR workers had higher
362 amounts of hydrocarbons than QL and QLBL workers (post-hoc LSD pairwise contrast, $p < 0.001$
363 for QLBL vs. QR, $p = 0.011$ for QL vs. QR and $p = 0.111$ for QL vs. QLBL). Post hoc analysis
364 revealed that hydrocarbon amounts also increased with age until day 7 at which point a plateau
365 was reached (post-hoc LSD pairwise contrast, $p < 0.05$ for comparisons of days 1-2 to 7-8, $p >$
366 0.05 for all later time points). The analysis of interaction between age and social condition
367 showed that differences between social conditions began on day 5 with the highest amounts of
368 hydrocarbons in QR workers and the lowest amounts in QLBL workers (post-hoc LSD pairwise
369 contrast, $p < 0.05$ for QR vs. QLBL, $p > 0.05$ for other comparisons, on days 11-12 $p < 0.05$ for all
370 comparisons).

371

372 Total secretion

373 The amount of DG total secretion was significantly affected by social condition and age with
374 significant interaction between the two factors, and covaried significantly with oocyte size
375 without significant interaction between oocyte size and social condition (GLMM, $F_{6,173}$=8.48, p
376 < 0.001 for age, $F_{2,173}$=4.78, p = 0.01 for social condition, $F_{11,172}$ = 2.06, p = 0.026 for age*social
377 condition interaction, $F_{1,174}$= 12.06, p = 0.001 for covariance with oocyte size, $F_{2,174}$ = 2.53, p =
378 0.082 for oocyte size*social condition interaction, data not shown). Overall, QR workers had
379 higher amounts of total secretion than QL and QLBL workers (post-hoc LSD pairwise contrast, p
380 = 0.002 for QLBL vs. QR, p = 0.032 for QL vs. QR and p = 0.237 for QL vs. QLBL). Post hoc
381 analysis revealed that the total secretion increased with age until day 7 at which point a plateau
382 was reached (post-hoc LSD pairwise contrast, p < 0.05 for comparisons of days 1-2 to 7-8, p >
383 0.05 for all later time points). The analysis of interaction between age and social condition
384 showed that differences between social conditions began on day 5 with the highest total
385 secretion in QR workers and the lowest amount of secretion in QLBL workers (post-hoc LSD
386 pairwise contrast, p < 0.05 for QR vs. QLBL, p > 0.05 for other comparisons, on days 11-12 p <
387 0.05 for all comparisons).
388
389 Overall, both ester and hydrocarbon amount increased with age but only the hydrocarbon
390 amount differed significantly between the social conditions and covaried with oocyte size.
391 Hydrocarbon amounts were highest in QR workers starting on days 5-6, in a similar fashion as the
392 total secretion, and ester proportions covary with oocyte size but fluctuate over age groups and
393 social conditions. To further examine the link between DG chemistry and the reproductive state
394 of workers, we performed a discriminant analysis with three categorial classifications of the
395 ovaries (see Methods for how categories were determined; Fig. 3). This analysis demonstrates
396 that either esters, alkenes or the entire secretion provides more group differentiation than
397 alkanes when workers are grouped according to ovarian activation.
398
399 Figure 3 - Linear discriminant analyses on Z-transformed relative peak areas of Dufour's gland
400 extracts of workers with three ovarian activation categories as the grouping variable. Each point
401 is the value from an individual bee. Panels **a-d** show the clustering generated by plotting the
402 first two linear discriminant functions using the esters, alkanes, alkenes, or all compounds.
403

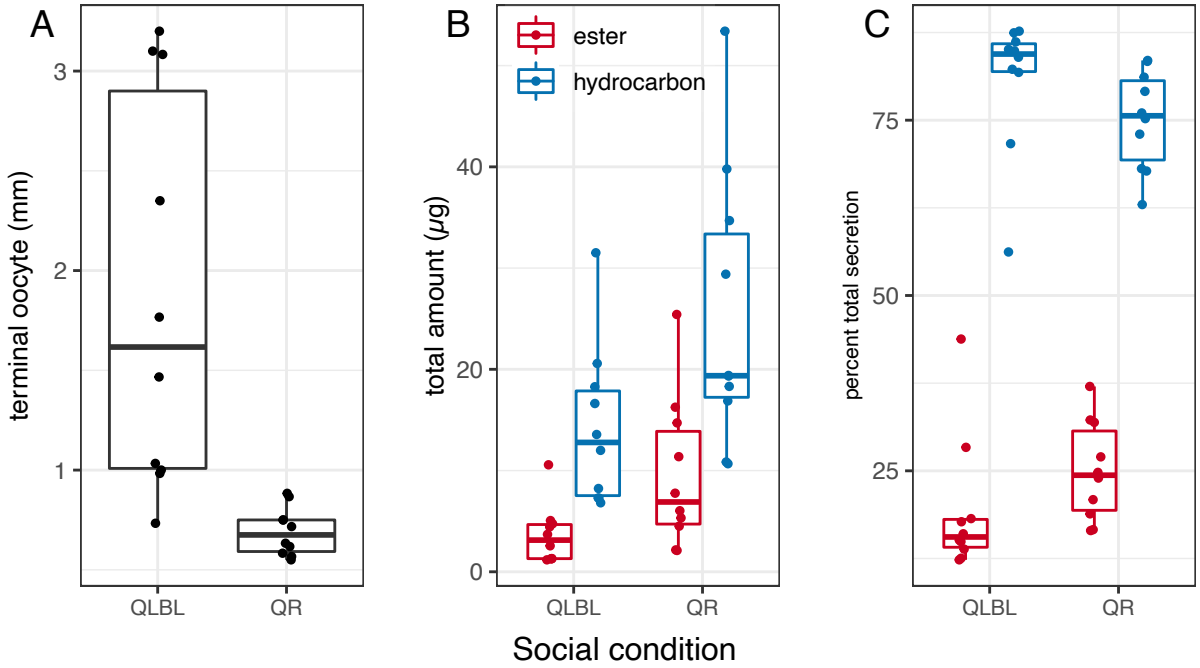


Examining the behavioral responses of *B. impatiens* workers to Dufour's gland extracts

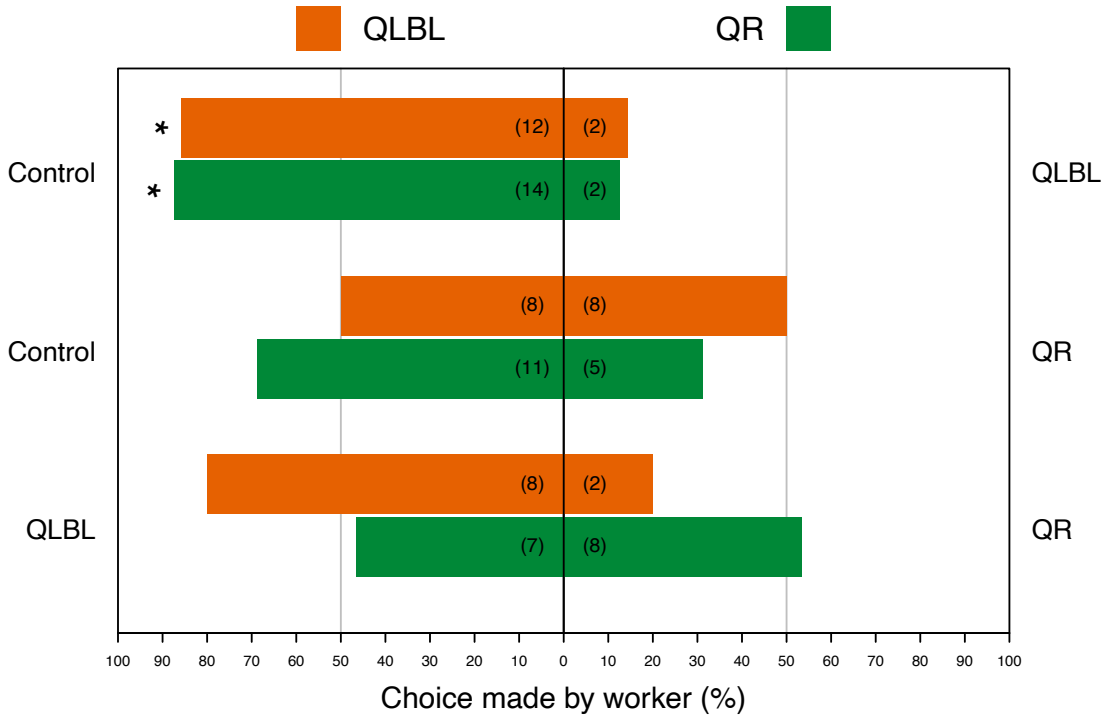
Due to the large impact of age we performed these experiments using same-aged workers at the age of 6-7 days from the two most distinct social conditions (QR and QLBL). Workers of this age showed significant differences in their ovary activation, amount and percent of esters and hydrocarbons (ovaries: Wilcoxon test, W = 4, p < 0.001; QR – 0.7 ± 0.04, QLBL – 1.9 ± 0.31; mean mm ± SE, n = 10), amount of esters (W = 0.8, p < 0.001; QR – 4.8 ± 1.1, QLBL – 0.9 ± 0.1; mean µg ± SE, n = 10), amount of hydrocarbons (W = 86, p = 0.005; QR – 14.4 ± 2.6, QLBL – 5.6 ± 1.1; mean µg ± SE, n = 10, percent esters (W = 79, p = 0.029; QR – 25.0 ± 2.2, QLBL – 19.3 ± 3.1; mean percent ± SE, n = 10; Fig. 4). In olfactometer bioassays, workers from both social conditions (QR, QLBL), given a choice between a callow worker DG extract (which served as a control) and QLBL DG extract, avoided QLBL DG extract (QLBL responders, n = 14, p = 0.01; QR responders n = 16, p = 0.004, binomial exact test; Fig. 5). When provided with a choice between callow extract vs. QR DG extract, workers of neither social conditions showed a significant preference (QLBL responder, n = 16, p = 1; QR responder, n = 16, p = 0.21). Similarly, when provided with a choice

between QLBL and QR DG extracts, neither showed a significant preference (QLBL responder, n = 15, p = 1). Both same-age QR and QLBL workers preferred extracts that represented lack of competition (i.e., callow or queenright workers). On average, QLBL workers at the age of 6-7 days had lesser amounts of esters and higher ovary activation (Fig. 4), and preferred the control over QLBL extract and QLBL over QR extract, while same-age QR workers had, on average, more esters and hydrocarbons and lower ovary activation (Fig. 4), and preferred the control over either QLBL or QR extracts.

Figure 4 - Reproductive state and Dufour's gland chemistry of 6-7 day old workers in queenless (QR) and queenless-broodless (QLBL)groups. (A) mean terminal oocyte size (mm), (B) mean µg of total esters and hydrocarbons ± SE (n = 10), and (C) mean percent of total esters and hydrocarbons ± SE (n = 10). Each data point is the value from an individual worker.



434 Figure 5 – The proportion of queenless brood-less (QLBL) and queenright workers (QR)
435 responding to 3 different combinations of stimuli, designated on the left and right of the plot:
436 control vs. QLBL DG extract, control vs. QR DG extract, and QLBL vs. QR DG extract. Control
437 stimuli consisted of callow worker DG extract. All stimuli were presented in the amount of 1
438 gland equivalent. The number of workers responding to each treatment is given in parenthesis
439 on each bar. Orange bars represent QLBL response bees, and green bars represent QR response
440 bees. Asterisks indicate significance (p <0.05) using exact binomial tests.



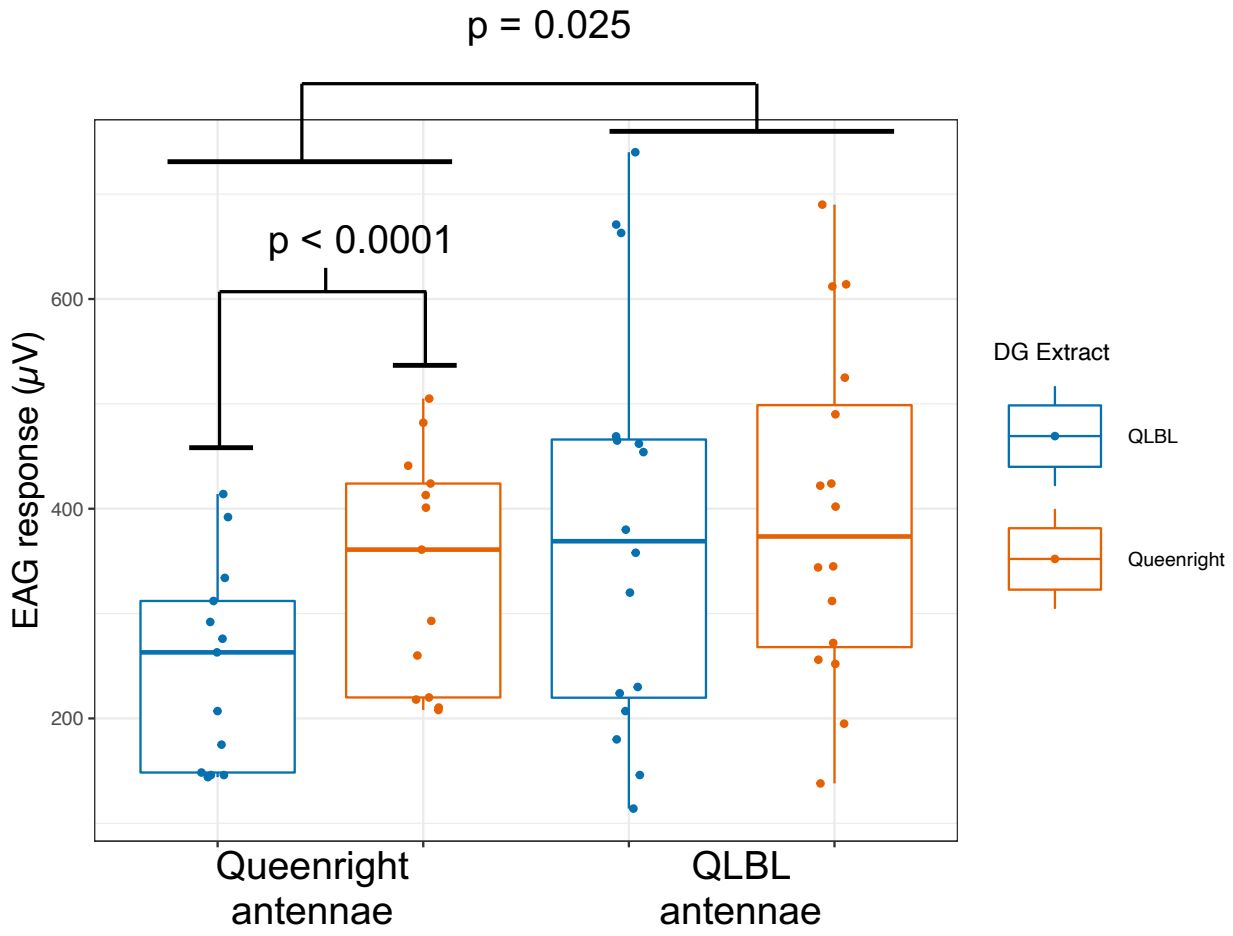
441

442 **Examining the antennal responses of *B. impatiens* workers to Dufour's gland extracts**

443 Electroantennogram recordings from antennae of 7-day-old workers of different social
444 conditions responding to QLBL or QR DG extracts (also from 7-day-old workers) revealed that
445 antennae of QLBL workers produced stronger responses, on average, than those of QR workers
446 ($p = 0.02$, Fig. 6). Within social condition, QR workers produced stronger responses to DG extract
447 from their own social condition than the QLBL extract ($p < 0.001$, Fig. 6), while QLBL workers
448 showed no difference in response based on the extract type ($p = 0.64$). Overall, QLBL that had,
449 on average, greater ovary activation and lesser amounts of esters (Fig. 4) exhibited stronger
450 responses than QR, and QR that had, on average, lower ovary activation and more esters were
451 more responsive to QR extracts.

452

453 Figure 6 – Electroantennogram responses (µV) of queenless brood-less and queenright worker
454 antennae to Dufour's gland extracts (1 worker equivalent) from queenless brood-less and
455 queenright workers. Blue and orange boxplots represent responses to queenless brood-less and
456 queenright Dufour's gland extracts, respectively. Asterisks indicate significance (* p < 0.05, ***
457 p < 0.001).



458

459

460 **Discussion**

461 The results of this study reveal the presence of caste-specific and physiology-specific compounds
462 in the DG of *B. impatiens* females. Our results further demonstrate that the DG secretion not only
463 contains important information about caste, age, social, and reproductive condition, but also that
464 the signaling properties of the gland contents are perceived and used by workers to discriminate
465 among workers. This discrimination suggests that workers are attracted to DG extracts of workers
466 that represent a lack of competition, and that QLBL workers, as opposed to QR workers, have
467 higher sensitivity to DG extracts, representing an increased investment in sensory capability.

468

469 The discriminant analysis of DG secretion showed clear differentiation of worker and queen
470 groups, highlighting the caste-specific chemistry of the gland. This separation was primarily
471 explained by worker-specific esters, gyne-specific esters, and queen-specific diterpene
472 compounds, although alkanes and, to some extent, alkenes contributed to some of the variability
473 (Fig. 1). The long chain esters that separate gynes from workers are also found in much lower
474 amounts in queens, pointing towards a signal that functions either in caste recognition by
475 workers, or sexual attraction of males. While the latter was not investigated in this study, caste
476 recognition by workers is supported by characteristic behaviors of workers towards gynes. For
477 example, although workers can be quite aggressive towards other workers or queens from a
478 different colony, they remain docile in the presence of gynes (Pers. obs., ND and EA).

479

480 The diterpene compounds that characterize the queen DG secretion are primarily known from
481 the labial glands of bumble bee males[44,45], where they are hypothesized to serve as sex-
482 pheromone components[46]. β-springene in particular has also been identified from the DG of a
483 braconid wasp[47], but the function it serves is unknown. Female sex pheromones in the related
484 species *B. terrestris* were identified from head and body surface extracts of gynes[48], but DG
485 components have not been investigated as sex pheromones. Previous research comparing the
486 DG secretion in unmated gynes of 5 species of European bumble bee found large variation in
487 long-chain wax esters, with *B. pascuorum* and *B. lucorum* possessing up to 5% of hexadecenyl
488 hexadecenoate or octadecyl octadec-9-enoate, while *B. hypnorum, B. lapidarius,* and *B. terrestris*
489 had none or less 1%[49]. Interestingly, diterpenes were not found in the DG of these species,
490 indicating that they may be species- and caste-specific. The DG's diterpene compounds in *B.*
491 *impatiens* could also function in caste recognition, a role that is often ascribed to cuticular
492 hydrocarbons[50,51]. The DG of the honey bee contains ~12 times more secretion in queens than
493 workers and contains caste specific compounds, leading previous researchers to hypothesize that
494 the gland deposits a signal of egg-maternity that distinguishes queen- from worker-laid eggs[52,53].
495 Caste-specific compounds, likely from the DG, have also been identified from the eggs of the
496 bumble bee *B. terrestris*[54]. In *B. impatiens*, it is unclear why both the queen and worker castes

497 would each need a unique set of compounds to differentiate egg-maternity, or why workers
498 would advertise egg maternity, because this is likely to result in oophagy[3], suggesting that these
499 compounds might have another as yet unknown signaling function.
500
501 Workers produced a series of dodecyl esters that were not detected in extracts from gynes or
502 mated queens (Fig S2, Table S2). These esters as well the hydrocarbon compounds in their DG
503 may be used as signals by conspecific queens, gynes, males or workers. While we only focused
504 on the signaling role of the secretion to other workers, the gland may contain multiple signals
505 targeting different individuals, as occurs with CHCs which signal both fertility and nestmate
506 identity in many social insects[51,55], or with the honey bee queen mandibular pheromone, which
507 functions as a primer pheromone to both workers and males, and a sex pheromone to males[56,57].
508 In *Bombus terrestris,* the main role of the DG worker-specific signals is in the interactions among
509 workers[8,21,34,58]. QR workers may gain from both producing a signal that conveys harmony and
510 respond to such a signal in order to maintain the social phase they are in (i.e., protect the queen
511 and ensure the production of future gynes), while QL workers, who enter a reproductive mode,
512 may gain from being tuned to signals that convey potential competition that may threaten the
513 fate of their eggs. By manipulating social condition to produce either QLBL reproductive workers
514 or QR non-reproductive workers, and then testing their perception and behavioral response to
515 DG signals from those same groups, we show that workers preferentially avoid bees of a non-
516 harmonious reproductive state (Fig. 5), with both QR and QLBL workers choosing callow over
517 QLBL extract, and being indifferent between control and QR extracts. As bees from both social
518 conditions were responding to aliquots of the same gland extract pooled from several colonies,
519 nestmate information was obscured and is unlikely to explain the differences in attraction.
520
521 In electroantennography experiments, antennae from QLBL workers produced stronger
522 responses than QR antennae (Fig. 6), suggesting that QLBL antennae are more sensitive to
523 perceiving DG signals. Queenlessness is known to affect olfactory development and performance
524 in the honey bee, where the worker olfactory system develops substantially during the first 6
525 days of life, and is strongly affected by the queen's presence[59,60]. Bumble bees and honey bees

526 have similar life span and 6-7 days is the same time period that workers in this study were kept
527 queenless. Additionally, in honey bee workers, queenlessness is associated with a reduction in
528 glomerular volume in the antennal lobe, and decreased learning of conditioned stimuli[61]. It is not
529 known if similar processes govern bumble bee worker development. Reproductively active 7-day-
530 old workers are likely capable of laying eggs and would benefit from detecting and avoiding the
531 queen, who would aggress the workers that challenge her reproductive monopoly. Additionally,
532 QR workers showed a reduced antennal response to QLBL extract in comparison to QLBL workers
533 in electroantennography experiments (Fig 6). This could indicate that QR bees are operating in
534 "harmony mode" within the colony with a higher threshold for detecting and aggressing other
535 individuals. It is not known at what level this differential response is regulated. QR and QLBL bees
536 could have differing numbers of olfactory receptors tuned to the DG compounds, or these
537 chemical signals could be interpreted at higher levels of signal integration and processing in the
538 brain.
539
540 Within workers, the composition of the DG secretion varied between social conditions, and the
541 amount of the secretion increased with age (Fig. 2), with differences in the amounts and
542 proportions of esters and hydrocarbons at certain age groups (eg, in 6-7-day-old QR and QLBL
543 workers; Fig. 4). While the broad qualitative composition of DG extracts in *B. impatiens* workers
544 is similar to related species of *Bombus*[8,54,62,63], the influence of social condition and reproductive
545 state on gland chemistry is different than the most closely studied species, *B. terrestris*. In *B.*
546 *terrestris* workers, the percentages of DG esters are negatively correlated with ovarian
547 activation[8,34], and the size of the gland is positively correlated with ovarian activation[64]. Here, we
548 demonstrate that in *B. impatiens*, the amounts of compounds of all chemical classes, including
549 esters, are positively correlated with age but affected by social condition at specific ages, while
550 the percent composition is more stable over time (Fig. 2B). This is distinctly different than the
551 case of esters in *B. terrestris* and indicates that differences in the amounts of compounds
552 observed at specific ages result from upregulation of the gland as a whole, instead of specific
553 components changing independently. In some systems, when a worker transitions into fertility,
554 their pheromonal secretion becomes more queenlike[33]. In *B. impatiens*, fertile worker secretions

555 do not mimic queen secretions, instead both maintain the production of compounds unique to
556 their own caste. Thus, the quantitative differences in DG components appears to represent a
557 fertility signal unique to the worker caste as compared to *B. terrestris,* where the wax esters are
558 only present in workers with inactivated ovaries and their absence fully mimics the queen
559 secretion[8]. This may require a more finely tuned threshold for aggression and help explain the
560 relatively docile conflict between queen and workers over reproduction in *B. impatiens*
561 compared with *B. terrestris*[65,66]. Similar processes may take place during the competition phase
562 when a large portion of the workers activate their ovaries. Furthermore, the DG esters also vary
563 between the two species, with dodecyl esters predominating in *B. impatiens* and octyl esters
564 predominating in *B. terrestris*[8]. Interestingly, dodecyl esters are also found in the labial glands of
565 *B. terrestris* females[67] whereas predominately octyl esters are found in the labial glands of *B.*
566 *impatiens* (Orlova M, Villar G, Hefetz, A, Millar J, Amsalem E, in preparation), presenting an
567 interesting case of inversion and suggesting a regulatory system that is shared but not identical
568 in different bumble bee species.
569
570 Thus, our data suggest that the DG contains multiple concurrent signals that contain information
571 about caste, age, social and reproductive condition. The presence of diterpene and ester
572 compounds, which are much less common than aliphatic alkanes and alkenes in insects, may
573 serve as additional channels with which to communicate this information. It is also possible that
574 the signal carries different information to different perceiving individuals. For instance, diterpene
575 compounds may signal "gyne" to conspecific workers and "mate" to conspecific males. Future
576 research should investigate where compounds from the DG can be found in a colony, whether
577 they are deposited onto eggs and/or the cuticular surface of the bee, and their role in worker-
578 worker, worker-queen and queen-male interactions.
579
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583

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758

759 **Author Contributions**

760 EA, AH, GV designed the experiments. GV and ND performed dissections, ovary measurements,

761 and gland extracts. ND and MO performed data and statistical analyses. GV performed
762 olfactometer and GC-EAD bioassays. ND and JM performed GC-MS analysis. ND and EA wrote
763 the manuscript. All authors provided feedback and edits.
764
765 **Additional Information**
766 Competing interests
767 The authors declare no competing interests.