Show me your Dufour's gland and I'll tell you who you are: 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, were produced in larger quantities by queenless, fertile workers at specific age groups as compared to queenright, 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] and by marking eggs to indicate their maternity, which prevents their consumption by "policing" workers[10–12]. In workers, chemical signals can indicate fertility status[13–15], which increases aggressive behavior towards fertile workers[16] or indicate sterility, which appease aggressive individuals[8,17]. Across social insects, these signals are chemically diverse and have multiple glandular origins[18,19].

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[20,21]. In solitary bees, the DG secretion functions primarily in waterproofing brood cells[22,23], although there is also evidence that it is the source of a sex pheromone in two parasitoids[24], and may serve as a nest-marking pheromone in certain solitary bee species[25,26]. In social bees, analyses of the DG secretion have suggested additional functions in social communication[27], such as signaling of reproductive status and the possible regulation of worker aggressive and foraging behaviors in honey bees and bumble bees[8,17,28,29].

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 latter half of the colony cycle, the competition phase, where workers initiate egg laying and produce males in competition with the queen[30]. In *Bombus terrestris,* the DG secretion varies with caste, reproductive state, age, and task[8,29]. 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

62 sterility[8]. There is also a negative correlation between the amount of esters a worker possesses
63 and the aggression directed toward her during worker-worker competition over reproduction,
64 suggesting an appeasement effect of these esters, in line with their hypothesized function as
65 sterility signals[17]. It is unknown if these signals are widespread in *Bombus*, or represents a unique
66 chemical signaling mechanism in *B. terrestris*. In the present study, we leverage our
67 understanding of *B. terrestris* chemical ecology in order to glean new insights into potential
68 shared or elaborated mechanisms mediating social organization across bumble bee species.
69
70 To examine the role of the gland from a signal production standpoint and discover if *B. impatiens*
71 females produce a social signal in the DG linked to their fertility status, we examined the chemical
72 composition of the secretion as a function of caste, age, social, and reproductive status. To
73 examine the signaling role of the gland from a sensory standpoint, we tested the behavioral and
74 antennal responses of workers, either queenless or queenright, to the DG secretion of either
75 queenless or queenright workers. The DG may carry multiple social signals which are caste or
76 social status dependent. Depending on what these roles are, DG compounds may induce
77 attraction or avoidance to facilitate cooperation or competition. Therefore, we hypothesize that
78 if workers produce a cooperative signal (e.g., sterile workers producing esters), a DG extract of
79 these workers will elicit higher attraction by cooperative workers in comparison with a control
80 extract, and if workers produce a fertility signals, a DG extract of these workers will elicit
81 avoidance in competing workers as compared to control. Additionally, we hypothesize that the
82 secretion from workers carrying a cooperative or competitive signal will elicit stronger antennal
83 responses in other workers, reflecting increased sensitivity towards compounds that may impact
84 their fitness.
85
86 **Materials and Methods**
87 *Bee colonies and sample preparation for chemical analysis*
88 Worker samples were collected from *B. impatiens* colonies (n = 8) that were obtained from
89 Koppert Biological Systems (Howell, Michigan, USA) or Biobest Canada Ltd. (Leamington, ONT).
90 Upon arrival to the laboratory, colonies were approximately 2-3 weeks old (based on first worker

91 emergence), with less than 30 workers each, a queen, and all stages of brood. Colonies were
92 maintained in nest-boxes at a constant temperature of 28–30°C and 60% relative humidity,
93 constant darkness and supplied *ad libitum* with a 60% sugar solution and honeybee-collected
94 pollen. These colonies were used as sources of newly-emerged workers that were sampled prior
95 to sexual production phase (ie, gynes and males). Newly-emerged workers were assigned to one
96 of three treatments: queenless (QL, n=70), queenless and brood-less (QLBL, n=70), and
97 queenright (QR, n=70) workers. QR workers were individually labeled and remained in their natal
98 colony in the presence of the queen until the day of collection while QR and QLBL workers were
99 housed in a plastic cage (11 cm diameter × 7 cm height) in groups of 3-6 workers without a queen.
100 Queenless groups of workers typically lay eggs within 6-8 days[31], and the presence of brood may
101 also affect worker reproduction. Therefore, in the QL groups, eggs laid by workers remained in
102 the cages, whereas in the QLBL groups, eggs laid by workers were removed daily. QR workers
103 were collected from young queenright colonies showing no signs of competition over
104 reproduction between the queen and the workers. Workers ranging in age from one to fourteen
105 days old (5/age group) were collected from all treatments. All workers were collected and stored
106 at −80° C until dissection.
107 Egg-laying queens (n=20) were obtained from full-sized colonies with >100 workers. All egg-laying
108 queens were actively producing female workers prior to sampling. Unmated queens (gynes) were
109 collected from 3 gyne-producing colonies. They were separated from their natal colonies upon
110 emergence to prevent mating, housed in small cages in groups of 3-5 gynes and sampled at 4 age
111 points: 3, 6, 10, and 14 days after emergence (5/age group, 20 queens in total).
112 Olfactometer and electrophysiology bioassays were conducted using 7-day old QR and QLBL
113 workers exposed to DG extracts of either 7-day old QR or QLBL workers, based on differences
114 observed in the chemical analysis and ovarian activation in these groups.
115
116 Dissection of bees
117 Ovaries were dissected under a stereomicroscope in distilled water, and the largest three
118 terminal oocytes across both ovaries (at least one from each ovary) were measured with an
119 eyepiece micrometer. The mean of these oocytes for each bee was used as a measure of ovary

120 activation. Mean terminal oocytes were used either as a continuous variable or to categorize
121 workers into three groups: inactive (<0.5mm), intermediate (0.5 - 2mm), and active (>2mm).
122 DGs from each individual were dissected and immediately immersed in hexane. Glands were
123 extracted for 48 hours after which each sample was evaporated and reconstituted in 100 µL
124 hexane spiked with an internal standard of 1 µg eicosane (C_{20}).
125
126 Chemical analysis
127 To identify compounds in the DG, extracts were analyzed on two separate mass spectrometers.
128 At UC Riverside, samples were analyzed on a Hewlett-Packard (H-P, now Agilent Technologies,
129 Santa Clara CA, USA) 6890 gas chromatograph (GC) equipped with a DB-17 mid-polarity column
130 (0.25 mm id x 30 m x 0.25 µm film thickness; J&W Scientific, Folsom CA, USA) interfaced to an H-
131 P 5973 mass spectrometer. The temperature program was as follows: 100˚C (0 min. hold) to
132 160˚C at 20˚C/min, then 4˚C/min to 280˚C (hold for 25 min). Injections were made in split-less
133 mode, with injector and transfer line temperatures of 280˚C. At Penn State, samples were
134 analyzed on an Agilent 7890A GC equipped with a HP-5ms column (0.25 mm id x 30 m x 0.25 µm
135 film thickness, Agilent) and interfaced to an Agilent 5975C mass spectrometer. Compounds were
136 tentatively identified based on diagnostic ions in the resulting spectra and retention indices, and
137 where possible, identifications were confirmed by matching retention times and mass spectra
138 with those of authentic standards.
139 Compounds in DG extracts were quantified on a Trace 1310 GC (Thermo Fisher, Waltham, MA
140 USA) equipped with a flame-ionization detector (FID) and a TG-5MS column (0.25 mm id x 30 m
141 x 0.25 µm film thickness; Thermo Fisher). The temperature program was as follows: 60˚C to 120˚C
142 at 15˚C/min followed by 120˚C to 300˚C (5m hold) at 4˚C/min. The injector port and FID were
143 held constant at 250˚C and 320˚C, respectively.
144
145 Olfactometer Bioassays
146 To examine if *B. impatiens* workers perceived the DG secretion by olfaction, and if their response
147 depended on reproductive or social state, bioassays were conducted using two-choice
148 olfactometers as previously described[32]. Olfactometers (see Fig. S1 for illustration) were

149 fashioned from plastic petri dishes (150 × 15 mm) with two equidistant holes (2 cm diameter) in
150 the bottom providing access to small plastic cups that held treatment or control stimuli (QR or
151 QLBL DG extract or control). Bees were prevented from returning to the main arena after a choice
152 was made via a 3 cm section of plastic straw glued to each hole. After lifting the petri dish lid
153 enough to allow entry, workers were placed in the center of the olfactometer and given 60 min
154 to make a choice. One gland equivalent of extract (100 µl total volume) was pipetted onto glass
155 coverslips and the solvent was allowed to evaporate before being introduced into the cups, which
156 were subsequently affixed to the olfactometer. Bioassays were conducted under a red light and
157 olfactometers were washed with soap and water and allowed to air dry before being reused.
158 Cups containing test stimuli, extracts and bees were only used once. All behavioral assays were
159 conducted in the rearing chamber between noon to 4 PM.
160 7-day old QR (n=39) or QLBL workers (n=48) were tested for their preference between 2 choices
161 in three bioassays: QR DG extract vs. callow DG extract, QLBL DG extract vs. callow DG extract,
162 and QR DG extract vs. QLBL DG extract. Overall, we conducted 122 bioassays (16-25 replicates
163 per treatment group and social condition) which resulted in 71.3% response rate. Bioassays
164 where bees did not respond within the time frame were not included in the analysis. Non-
165 responder bees were not associated with any specific treatment (proportion test, $\chi^2 = 0.16$, $p =$
166 0.69, 25.9% and 30.9% of QL and QR non-responders, respectively). Callow DG extract was used
167 as control as it is undifferentiated into a specific chemical profile, being mostly devoid of potential
168 signal molecules, but went through the same extraction process as 7-day old worker DG,
169 capturing methodological noise that a solvent control would not. In addition, the preference to
170 a solvent control and callow extract were not significantly different when compared directly
171 (binomial exact test, $p = 0.77$, $n = 12$, 20% non-responders). In all bioassays, extracts were
172 presented to workers as a dose of 1-bee equivalent. Extracts were prepared as described above
173 for the chemical analyses, pooled, and the concentration was adjusted to 1 gland equivalent per
174 100 µL hexane.
175
176 <u>Electroantennogram recordings</u>

177 Odor cartridges were created using 7-day old worker DG extracts, either QR or QLBL, as follows.
178 1 DG equivalent per 100 µL of hexane (DG extracts were prepared as above) was administered
179 onto strips of Whatman filter paper. After the solvent had evaporated, the filter paper strips were
180 inserted into glass pipettes and sealed with aluminum foil until use. A "blank" control consisting
181 of 100 µL of solvent was allowed to evaporate from the filter paper before being placed and
182 sealed in the cartridge. For the electroantennograms (QR n=13, QLBL n=16), the right antenna
183 was excised and the tip snipped with a fine scissors prior to mounting on a quadroprobe
184 electroantennogram system with Spectra 360 electroconductive gel[33]. A constant airflow of
185 charcoal-purified humidified air was passed across the antenna through a 10 mm glass tube for
186 the duration of each experiment. Each antenna was allowed to reach a steady baseline before
187 starting the experiment, at which point the test odorants were delivered into a steady airstream
188 using the odor cartridges in conjunction with a stimulus flow controller (Syntech, Hilversum,
189 Netherlands). A 40 ml/s air pulse was pushed through the cartridge for 0.05 seconds, effectively
190 delivering 2 ml of volatiles into the air stream and across the antenna. Responses to each stimulus
191 were amplified using a quadroprobe, 10x pre-amplifier (Syntech, Netherlands) and further
192 amplified 10x before being recorded and analyzed on a desktop computer using custom software
193 (as in[34]). Odorants from control, QLBL, or QR extracts were presented to each antenna 30 seconds
194 apart, after the antennal response had returned to baseline activity. Responses to a final blank
195 puff were subtracted from the responses to the test odorants and the corrected values were used
196 in analyses. Each worker antenna received all treatments, and each odor cartridge was used
197 twice.
198
199 Statistical analysis
200 Statistical analyses were performed using R (version 3.6.1) in RStudio (version 1.2.1335). To
201 examine differences in the DG secretion between females of differing caste (queen, workers) and
202 social conditions (QR, QL, QLBL worker groups), linear discriminant analyses (LDA) were
203 performed using Z-transformed relative peak areas as predictor variables, and social condition,
204 caste, or age as grouping variables. Only peaks > 0.5% of the total peak area were used. These
205 selected peaks were then re-standardized to 100% and transformed using Aitchinson's

206 formula [Zij = ln[Yij/g(Yj)][35], to reduce collinearity prior to multivariate analysis[36,37]. For peaks that
207 were not present in all groups, a small nominal value was added as the transformation cannot be
208 calculated with zero values. In some cases, poorly separated clusters of peaks prevented accurate
209 integration, and after mass spectral confirmation that these were of the same compound class
210 (e.g. esters), they were integrated together as a "complex" and used as a single variable.
211 Amounts of DG compounds and oocyte size were not normally distributed (Shapiro-Wilks test p
212 < 0.05) and were therefore analyzed with a generalized linear model (GLM) using the R package
213 lme4. To examine the effect of social condition and age on worker oocyte size, social condition
214 and age were used as fixed effects and colony as a random effect, with a gaussian distribution
215 and log as link function. When two numeric independent variables were included in the model,
216 these were centered and scaled to allow comparison of their coefficients. To examine the effect
217 of social condition, age, and oocyte size on the total amount of esters in worker DGs, those terms
218 were used as fixed effects, and colony as a random effect. Wilcoxon tests were used to compare
219 terminal oocyte size, and ester and hydrocarbon amounts between QLBL and QR 7-day workers.
220 Choice data from olfactometer bioassays were analyzed using exact binomial tests, calculating
221 two-tailed probabilities for a difference in the proportion of choices against the null hypothesis
222 of 50%, or no preference. The average strength of antennal responses from electroantennogram
223 recordings were compared using t-tests when comparing responses based on antennal social
224 condition, and paired t-tests when comparing antennal responses within social condition and
225 between extract type. Significance was evaluated at the $\alpha = 0.05$ level.
226
227 **Results**
228 **Chemistry of *Bombus impatiens* Dufour's gland**
229 DG extracts and the average oocyte size were analyzed in 210 workers (5/age group, ages 1-14
230 days for 3 treatment groups), 20 unmated gynes (5/age group, ages 3, 6, 10, 14 days), and 20
231 active queens (4-15 weeks from the emergence of the first worker). During analysis, we
232 discovered 11 outlier worker samples, which were excluded from further analyses. One QR
233 worker had fully activated ovaries at 8 days of age, which was a clear outlier, and 10 QL workers
234 on days 9 and 10 sharing the same 2 cages and from the same colony had unusually undeveloped

235 ovaries which likely resulted from a shared effect related to rearing conditions (e.g.,
236 underfeeding) or maternal colony. Thus, 199 workers were included in subsequent analyses.
237 Overall, GC-FID analysis of *B. impatiens* DG extracts in females revealed 98 compounds comprised
238 of aliphatic alkanes (C_{21}-C_{31}), alkenes, wax esters, and diterpenes (Fig. S2, Table S1).
239
240 **Examining caste specificity in Dufour's gland secretion**
241 The DGs of different castes of *B. impatiens* contain compounds that are both queen-specific and
242 worker-specific, in addition to linear alkanes and alkenes found in both castes (Fig S2). Gynes and
243 queens differed from workers based on the presence of the diterpenes ß-springene and isomers,
244 which were absent in workers. Conversely, three dodecyl esters (dodecyl octanoate, dodecyl
245 decanoate, and dodecyl dodecanoate), and hexadecyl decanoate were specific to workers (Fig
246 S2). In addition, workers produced large quantities of a fifth ester, dodecyl oleate, which was
247 present only in trace amounts in gynes. In gynes and queens, β-springene accounted for 3.2% ±
248 0.19 and 1.3% ± 0.33 (mean ± SE, n=20), on average, of the total secretion, respectively. The
249 worker-specific esters comprised, on average, 1.0% ± 0.07, 2.6% ± 0.08, 1.8% ± 0.08, 1.5% ± 0.13,
250 and 5.1% ± 0.28 (mean ± SE, n=199) of the total secretion across workers of all ages and
251 treatments, respectively. Long chain esters (tentatively identified as terpene esters 6 and 7)
252 comprised, on average, 1.2% ± 0.18 and 2.2% ± 0.26 (mean ± SE, n=20) of the gyne secretion,
253 while only being detected in trace amounts in workers, and at <1% in DG extracts of queens.
254 Discriminant analyses using either all compounds or specific chemical classes (esters, alkanes,
255 and alkenes) as predictor variables showed that esters enabled differentiation between active
256 queens, gynes, and workers with similar explanatory power as the analyses using all compounds
257 (Fig 1). Figure 1 shows plots of the data according to the first two linear discriminant functions
258 based on the compound class used as predictor variables. Of the worker esters, dodecyl
259 decanoate provided the greatest separation between queen and worker castes along LD1
260 (coefficient of LD1 1.71, % variation explained = 93%), while of the gyne esters, terpene esters 6
261 and 7 accounted for the most variability along LD2 (coefficients of LD2 0.94 and -1.22
262 respectively, % variation explained = 7%).
263

Examining the effect of age, social, 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 and tested the effects of worker age, social conditions, and reproductive status) on their DG composition. GLM models were fit using social condition (categorical variable with 3 levels: QR, QL, QLBL), age (as a scaled continuous variable, days 1-14 after eclosion), and ovarian activation (as a scaled continuous variable, 0-4 mm as measured by the average terminal oocyte size), and interactions between these variables when significant.

Reproductive Status. Ovarian activation increased with age in all treatment groups. However, workers in the QR social condition progressed at a slower pace compared to the QL and QLBL groups, which was especially evident during days 7-12 (Fig. 2A). There was a significant positive effect of ovarian activation on the amount of the total DG secretion (GLM estimate = 0.35, SE = 0.09, t-value = 4.02, $p < 0.0001$), and total hydrocarbons (GLM estimate = 0.22, SE = 0.07, t-value = 3.01, $p = 0.003$), but not total esters ($p = 0.94$) (Figs 2B & 2C. The total secretion is the sum of hydrocarbons and esters and therefore not shown separately).

Social condition. There was no significant effect of social condition on the amount of total DG secretion (QLBL GLM estimate = -0.09, SE = 0.10, t-value = -0.89, $p = 0.37$; QR GLM estimate = -0.11, SE = 0.09, t-value = -1.22, $p = 0.22$), or amount of total hydrocarbons (QLBL GLM estimate = -0.07, SE = 0.13, t-value = -0.70, $p = 0.48$; QR GLM estimate = -0.16, SE = 0.10, t-value = -1.68, $p = 0.09$). There was, however, a significant positive effect of social condition on the amount of esters in the QR (GLM estimate = 3.99, SE = 1.22, t-value = 3.25, $p = 0.01$), and QLBL groups (GLM estimate = 3.55, SE = 1.2, t-value = 2.94, $p = 0.003$) with higher amounts of esters produced by QR and QLBL workers vs. QL workers.

292 *Age*. There was a significant positive effect of age on the total DG secretion (GLM estimate = 0.47,
293 SE = 0.08, t-value = 6.18, $p < 0.0001$), the total amount of hydrocarbons (GLM estimate = 0.4, SE
294 = 0.07, t-value = 5.66, $p < 0.0001$), and the total amount of esters (GLM estimate = 0.37, SE =
295 0.09, t-value = 4.28, $p < 0.001$). There were positive effects of age (GLM estimate = 0.116, SE =
296 0.009, t-value = 12.27, $p < 0.0001$) and the QLBL social condition (GLM estimate = 0.429, SE =
297 0.075, t-value = 5.71, $p < 0.0001$) on worker ovarian activation (Fig. 2A). All variables tested in
298 this study were affected by age, demonstrating the need to control for age in order to understand
299 the signaling role of the glandular secretion in females.
300
301 Overall, the reproductive state of workers was positively correlated with the total secretion,
302 hydrocarbons and ester amounts, while the social condition was associated mostly by the ester
303 amounts. To further examine the link between DG chemistry and the reproductive state of
304 workers, we performed a discriminant analysis with three categorial classifications of the ovaries
305 (see Methods for how categories were determined; Fig. 3). This analysis demonstrates that either
306 esters or the entire secretion provide more group differentiation than alkenes or alkanes when
307 workers are grouped according to ovarian activation.
308
309 **Examining the behavioral responses of *B. impatiens* workers to Dufour's gland extracts**
310 The largest differences in ovary activation, amounts of esters and hydrocarbons between the two
311 most distinct social conditions (QR and QLBD) were at the age 6-7 days (ovaries: Wilcoxon test,
312 W = 4, $p < 0.001$; QR – 0.7 ± 0.04, QLBL – 1.9 ± 0.31; mean mm ± SE, n = 10), esters (W = 0.8, $p <$
313 0.001; QR – 4.8 ± 1.1, QLBL – 0.9 ± 0.1; mean µg ± SE, n = 10), and hydrocarbons (W = 86, p =
314 0.005; QR – 14.4 ± 2.6, QLBL – 5.6 ± 1.1; mean µg ± SE, n = 10; Fig. 4). Therefore, this time point
315 was chosen for the behavioral and antennal assays. In olfactometer bioassays, workers from both
316 social conditions (QR, QLBL), given a choice between a callow worker DG extract (which served
317 as a control) and QLBL DG extract, avoided QLBL DG extract (QLBL responders, n = 14, p = 0.01;
318 QR responders n = 16, p = 0.004, binomial exact test; Fig. 5). When provided with a choice
319 between callow extract vs. QR DG extract, workers of neither social condition showed a
320 significant preference (QLBL responder, n = 16, p = 1; QR responder, n = 16, p = 0.21). Similarly,

321 when provided with a choice between QLBL and QR DG extracts, neither showed a significant
322 preference (QLBL responder, n = 15, p = 1). Both 7-day old QR and QLBL workers preferred
323 extracts that represented lack of competition (i.e. callow or queenright workers). Seven day old
324 QLBL workers that had, on average, lesser amounts of esters and higher ovary activation (Fig. 4),
325 preferred the control over QLBL extract and QLBL over QR extract, while 7-day old QR that had,
326 on average, more esters and hydrocarbons and lower ovary activation (Fig. 4), preferred the
327 control over either QLBL or QR extracts.
328
329 **Examining the antennal responses of *B. impatiens* workers to Dufour's gland extracts**
330 Electroantennogram recordings from antennae of 7-day-old workers of different social
331 conditions responding to QLBL or QR DG extracts (also from 7-day-old workers) revealed that
332 antennae of QLBL workers produced stronger responses, on average, than those of QR workers
333 ($p = 0.02$, Fig. 6). Within social condition, QR workers produced stronger responses to DG extract
334 from their own social condition than the QLBL extract ($p < 0.0001$, Fig. 6), while QLBL workers
335 showed no difference in response based on the extract type ($p = 0.64$). Overall, QLBL that had,
336 on average, greater ovary activation and lesser amounts of esters (Fig. 4) exhibited stronger
337 responses than QR, and QR that had, on average, lower ovary activation and more esters were
338 more responsive to QR extracts.
339
340 **Discussion**
341 The results of this study reveal the presence of caste-specific and physiology-specific compounds
342 in the DG of *B. impatiens* females. Our results further demonstrate that the DG secretion not only
343 contains important information about caste, age, social, and reproductive condition, but also that
344 the signaling properties of the gland contents are perceived and used by workers to discriminate
345 among workers. This discrimination suggests that workers are attracted to DG extracts of workers
346 that represent a lack of competition, and that QLBL workers, as opposed to QR workers, have
347 higher sensitivity to DG extracts, representing an increased investment in sensory capability.
348

349 The discriminant analysis of DG secretion showed clear differentiation of worker and queen
350 groups, highlighting the caste-specific chemistry of the gland. This separation was primarily
351 explained by worker-specific esters, gyne-specific esters, and queen-specific diterpene
352 compounds, although alkanes and, to some extent, alkenes contributed to some of the variability
353 (Fig. 1). The long chain esters that separate gynes from workers are also found in much lower
354 amounts in active queens, pointing towards a signal that functions either in caste recognition by
355 workers, or sexual attraction of males. While the latter was not investigated in this study, caste
356 recognition by workers is supported by characteristic behaviors of workers towards gynes. For
357 example, although workers can be quite aggressive towards other workers or active queens from
358 a different colony, they remain docile in the presence of gynes (Pers. obs., ND and EA).
359
360 The diterpene compounds that characterize the queen caste DG are primarily known from the
361 labial glands of bumble bee males, where they are hypothesized to serve as sex-pheromone
362 components[38]. Female sex pheromones were identified from head and body surface extracts of
363 gynes[39], but DG components have not been investigated as sex pheromones. Previous research
364 comparing the DG secretion in unmated gynes of 5 species of European bumble bee found large
365 variation in long-chain wax esters, with *B. pascuorum* and *B. lucorum* possessing up to 5% of
366 hexadecenyl hexadecenoate or octadecyl octadec-9-enoate, while *B. hypnorum, B. lapidarius,*
367 and *B. terrestris* had none or less 1%[40]. Interestingly, diterpenes were not found in the DG of
368 these species, indicating that they may be species and caste specific. The DG's diterpene
369 compounds in *B. impatiens* could also function in caste recognition, a role that is often ascribed
370 to cuticular hydrocarbons[41,42]. The DG of the honey bee contains ~12 times more secretion in
371 queens than workers and contains caste specific compounds, leading previous researchers to
372 hypothesize that the gland deposits a signal of egg-maternity that distinguishes queen- from
373 worker-laid eggs[43,44]. Caste-specific compounds, likely from the DG, have also been identified
374 from the eggs of the bumble bee *B. terrestris*[45]. In *B. impatiens*, it is unclear why both the queen
375 and worker castes would each need a unique set of compounds to differentiate egg-maternity,
376 or why workers would advertise egg maternity, because this is likely to result in oophagy[3],
377 suggesting that these compounds might have another as yet unknown signaling function.

Workers produced a series of dodecyl esters that were not detected in extracts from gynes or active queens (Fig S2, Table S1). A signal is defined as a trait, structure, or behavior that evolved to convey specific information to, and elicit a response from, a receiver, increasing both the emitter's and receiver's overall fitness[46,47]. Because worker signals are specific to workers and are detected by other workers, they must benefit workers. We did not focus on the ability of queens, gynes, or males to differentiate workers based on their DG signals, but the secretion may contain multiple signals targeting different individuals, as occurs with CHCs which signal both fertility and nestmate identity in many social insects[42,48], or with the honey bee queen mandibular pheromone, which functions as a primer pheromone to both workers and males, and a sex pheromone to males[49,50]. The worker-specific signal could play a role in the interactions among workers, as in *Bombus terrestris*[8,17,29,51], or have an additional unknown signaling role. The benefit of the signal to the receiver in the interactions between workers also may change according to the social condition of the workers. QR workers may gain from both producing a signal that conveys harmony and respond to such a signal in order to maintain the social phase they are in (i.e., protect the queen and ensure the production of future gynes), while QL workers, who enter a reproductive mode, may gain from being tuned to signals that convey potential competition that may threaten the fate of their eggs. By manipulating social condition to produce either QLBL reproductive workers or QR non-reproductive workers, and then testing their perception and behavioral response to DG signals from those same groups, we show that workers preferentially avoid bees of a non-harmonious reproductive state (Fig. 5), with both QR and QL workers choosing callow over QL extract, and being indifferent between control and QR extracts.

In electroantennography experiments, antennae from QLBL workers produced stronger responses than QR antennae (Fig. 6), suggesting that QLBL antennae are more sensitive to perceiving DG signals. Queenlessness is known to affect olfactory development and performance in the honey bee, where the worker olfactory system develops substantially during the first 6 days of life, and is strongly affected by the queen's presence[52,53]. Bumble bees and honey bees have similar life span and 6-7 days is the same time period that workers in this study were kept

407 queenless. Additionally, in honey bee workers, queenlessness is associated with a reduction in
408 glomerular volume in the antennal lobe, and decreased learning of conditioned stimuli[54]. It is not
409 known if similar processes govern bumble bee worker development. Reproductively active 7-day-
410 old workers are likely capable of laying eggs and would benefit from detecting and avoiding the
411 queen, who would aggress the workers that challenge her reproductive monopoly. Additionally,
412 QR workers showed a reduced antennal response to QLBL extract in comparison to QLBL workers
413 in electroantennography experiments (Fig 6). This could indicate that QR bees are operating in
414 "harmony mode" within the colony with a higher threshold for detecting and aggressing other
415 individuals. It is not known at what level this differential response is regulated. QR and QLBL bees
416 could have differing numbers of olfactory receptors tuned to the DG compounds, or these
417 chemical signals could be interpreted at higher levels of signal integration and processing in the
418 brain.
419
420 Within workers, the composition of the DG secretion varied between social conditions, and the
421 amount of the secretion increased with age (Fig. 2), with differences in the amounts of esters and
422 hydrocarbons at certain age groups (eg, in 6-7-day-old QR and QLBL workers; Fig. 4). While the
423 broad qualitative composition of DG extracts in *B. impatiens* workers is similar to related species
424 of *Bombus*[8,45,55,56], the influence of social condition and reproductive state on gland chemistry is
425 different than the most closely studied species, *B. terrestris*. In *B. terrestris* workers, the
426 percentages of DG esters are negatively correlated with ovarian activation[8,29], and the size of the
427 gland is positively correlated with ovarian activation[57]. Here, we demonstrate that in *B.*
428 *impatiens*, the amounts of compounds of all chemical classes, including esters, are positively
429 correlated with age and ovarian activation, regardless of social condition, while the percent
430 composition is stable over time (Fig. S3). This indicates that differences in the amount of
431 compounds observed at specific ages result from upregulation of the gland as a whole, instead
432 of specific components changing independently. In some systems, when a worker transitions into
433 fertility, their pheromonal secretion becomes more queenlike[28]. In *B. impatiens*, fertile worker
434 secretions do not mimic queen secretions, instead both maintain the production of compounds
435 unique to their own caste. Thus, the quantitative differences in DG components appears to

436 represent a more subtle fertility signal than in *B. terrestris,* where the wax esters are only present
437 workers with inactivated ovaries[8]. This may require a more finely tuned threshold for aggression
438 and help explain the relatively docile conflict between queen and workers over reproduction of
439 *B. impatiens* compared with *B. terrestris*[58,59]. Furthermore, the DG esters also vary between the
440 two species, with dodecyl esters predominating in *B. impatiens* and octyl esters predominating
441 in *B. terrestris*[8]. Interestingly, dodecyl esters are also found in the labial glands of *B. terrestris*
442 females[60] whereas predominately octyl esters are found in the labial glands of *B. impatiens*
443 (Orlova M, Villar G, Hefetz, A, Millar J, Amsalem E, in preparation), presenting an interesting case
444 of inversion and suggesting a regulatory system that is shared but not identical in different
445 bumble bee species.
446
447 Thus, our data suggest that the DG contains multiple concurrent signals that contain information
448 about caste, age, social and reproductive condition. The presence of diterpene and ester
449 compounds, which are much less common than aliphatic alkanes and alkenes in insects, may
450 serve as additional channels with which to communicate this information. It is also possible that
451 the signal carries different information to different perceiving individuals. For instance, diterpene
452 compounds may signal "gyne" to conspecific workers and "mate" to conspecific males. Future
453 research should investigate where compounds from the DG can be found in a colony, whether
454 they are deposited onto eggs and/or the cuticular surface of the bee, and their role in worker-
455 worker, worker-queen and queen-male interactions.
456
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463

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Author Contributions

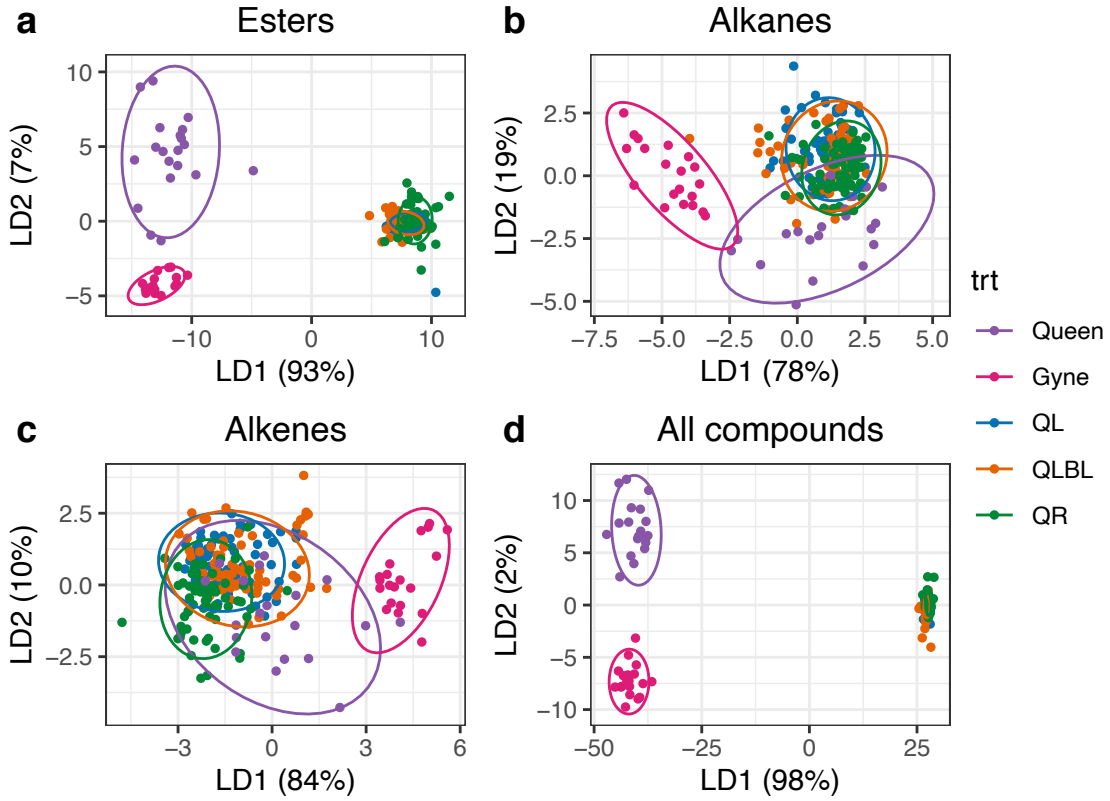
EA, AH, GV designed the experiments. GV and ND performed dissections, ovary measurements, and gland extracts. ND performed data and statistical analyses. GV performed olfactometer and GC-EAD bioassays. ND and JM performed GC-MS analysis. ND, and EA wrote the manuscript. All authors provided feedback and edits.

Additional Information

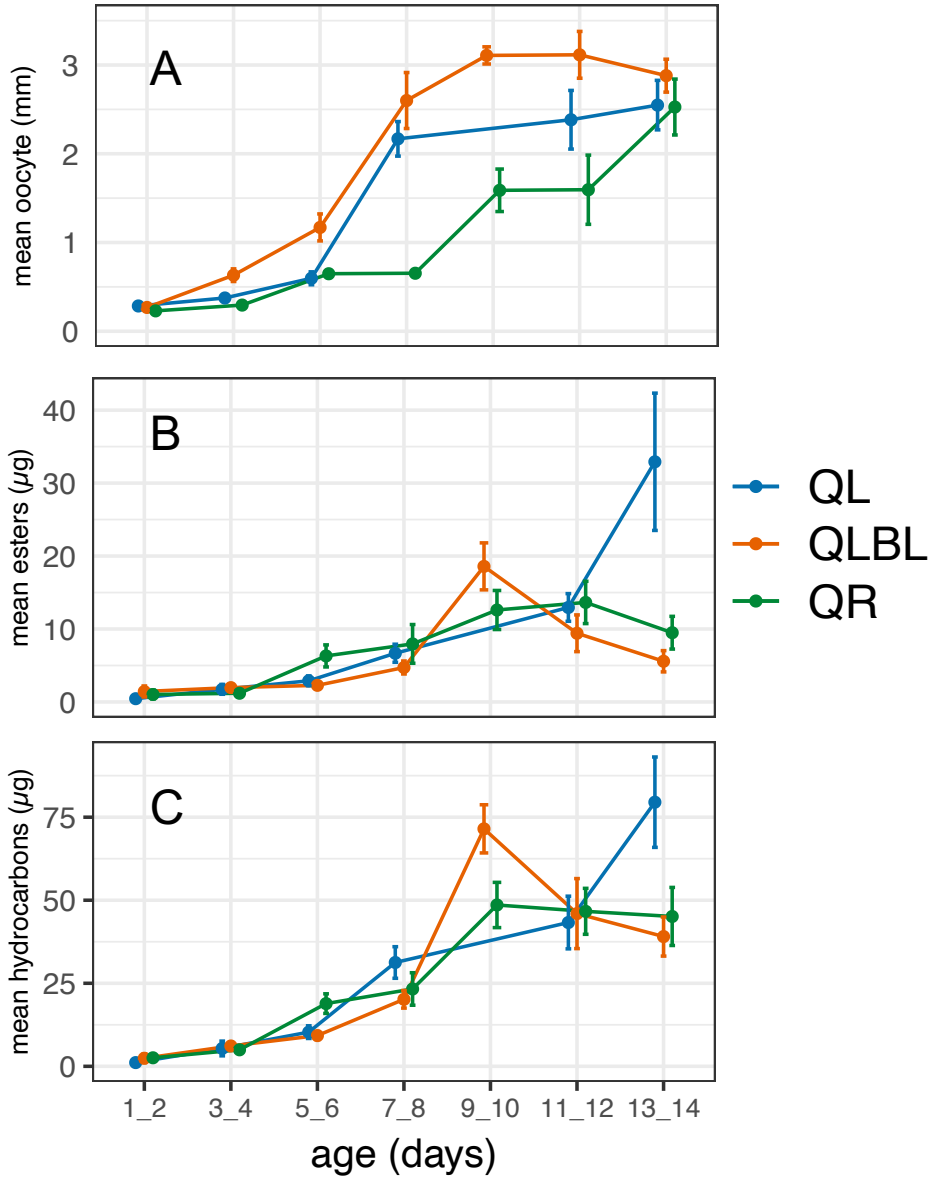
Competing interests

The authors declare no competing interests.

Figure 1 - Linear discriminant analyses on Z-transformed relative peak areas of Dufour's gland extracts of workers of three social condition treatment groups (queenright – QR, queenless - QL, and queenless, brood-less - QLBL), unmated gynes, and active queens, with caste/treatment as the grouping variable. Each point is an individual bee. Panels **a-d** shows the clustering generated by plotting the first two linear discriminant functions when using only the esters, alkanes, alkenes, or all compounds.



632 Figure 2 – Change in worker reproductive state and Dufour's gland chemistry as a function of
633 age (1-14 days post emergence) and social condition: queenright (QR), queenless (QL), and
634 queenless, brood-less (QLBL) workers. (A) mean terminal oocyte length (mm), (B) mean µg of
635 total esters ± SE, and (C) mean µg of total hydrocarbons ± SE. Data are grouped into two-day
636 bins and represent the mean value of 10 workers per age group.



637

Figure 3 - Linear discriminant analyses on Z-transformed relative peak areas of Dufour's gland extracts of workers with three ovarian activation categories as the grouping variable. Each point is the value from an individual bee. Panels **a-d** show the clustering generated by plotting the first two linear discriminant functions when using only the esters, alkanes, alkenes, or all compounds.

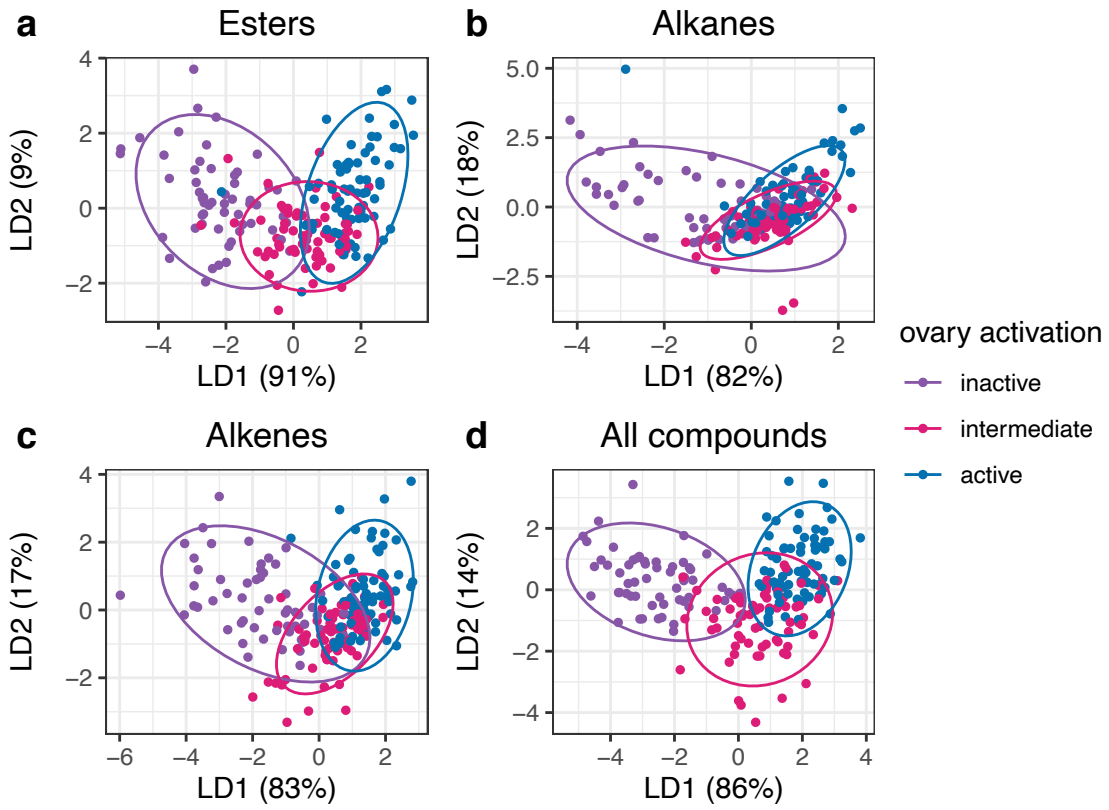
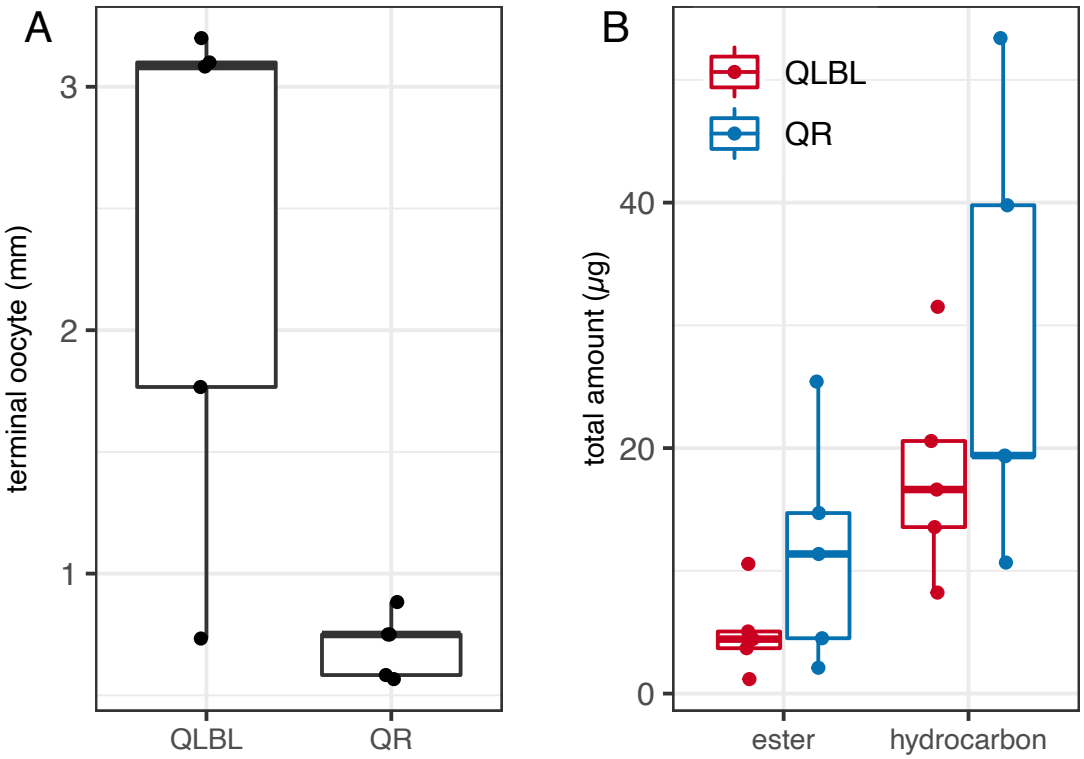


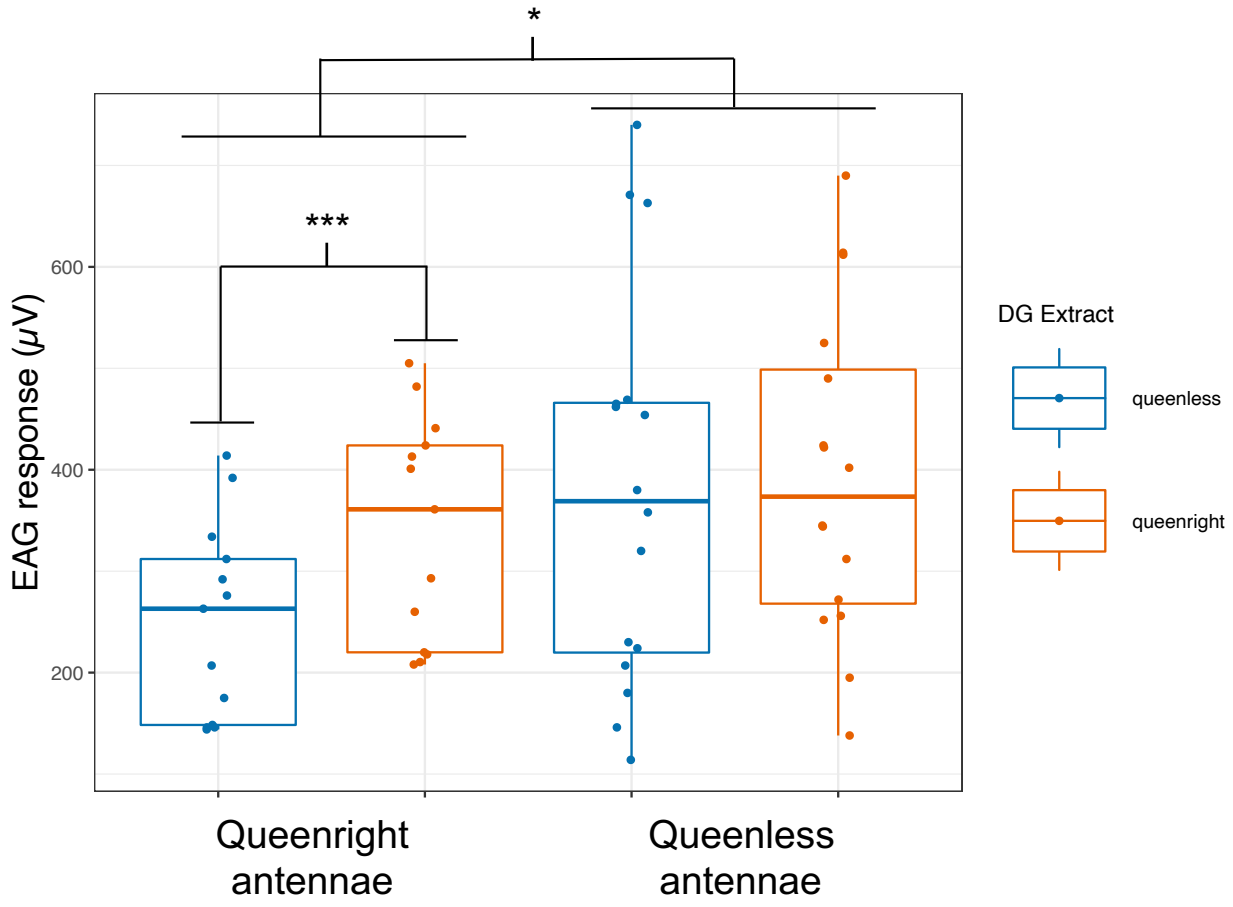
Figure 4 - Change in 6- and 7-day-old worker reproductive state and Dufour's gland chemistry as a function of social condition in queenless (QR) and queenless-broodless (QLBL) workers. (A) mean terminal oocyte length (mm), (B) mean µg of total esters and hydrocarbons ± SE (n = 10). Each data point is the value from an individual worker.



651 Figure 5 – The proportion of queenless brood-less (QLBL) and queenright workers (QR)
652 responding to 3 different combinations of stimuli, designated on the left and right of the plot:
653 control vs. QLBL DG extract, control vs. QR DG extract, and QLBL vs. QR DG extract. Control
654 stimuli consisted of callow worker DG extract. All stimuli were presented in the amount of 1
655 gland equivalent. The number of workers responding to each treatment is given in parenthesis
656 on each bar. Blue bars represent QLBL response bees, and orange bars represent QR response
657 bees. Asterisks indicate significance (p <0.05) using exact binomial tests.
658

659

660 Figure 6 – Electroantennogram responses (µV) of queenless broodless and queenright worker
661 antennae to Dufour's gland extracts (1 worker equivalent) from queenless broodless and
662 queenright. Blue and orange boxplots represent responses to queenless broodless and
663 queenright Dufour's gland extracts, respectively. Asterisks indicate significance (* $p < 0.05$, ***
664 $p < 0.0001$).
665



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