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Author for correspondence:
Tom Wenseleers
e-mail: tom.wenseleers@bio.kuleuven.be

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Honeybees possess a structurally diverse and functionally redundant set of queen pheromones

Sarah A. Princen[1], Ricardo Caliari Oliveira[1], Ulrich R. Ernst[1,2,3], Jocelyn G. Millar[4], Jelle S. van Zweden[1] and Tom Wenseleers[1]

[1]Department of Biology, KU Leuven, Laboratory of Socioecology and Social Evolution, Leuven, Belgium
[2]Department of Biology, KU Leuven, Functional Genomics and Proteomics Group, Leuven, Belgium
[3]Institute for Evolution and Biodiversity, University of Münster, Molecular Evolution and Sociobiology Group, Münster, Germany
[4]Departments of Entomology and Chemistry, University of California, Riverside, CA 92521, USA

URE, 0000-0002-6330-5341; TW, 0000-0002-1434-861X

Queen pheromones, which signal the presence of a fertile queen and induce workers to remain sterile, play a key role in regulating reproductive division of labour in insect societies. In the honeybee, volatiles produced by the queen's mandibular glands have been argued to act as the primary sterility-inducing pheromones. This contrasts with evidence from other groups of social insects, where specific queen-characteristic hydrocarbons present on the cuticle act as conserved queen signals. This led us to hypothesize that honeybee queens might also employ cuticular pheromones to stop workers from reproducing. Here, we support this hypothesis with the results of bioassays with synthetic blends of queen-characteristic alkenes, esters and carboxylic acids. We show that all these compound classes suppress worker ovary development, and that one of the blends of esters that we used was as effective as the queen mandibular pheromone (QMP) mix. Furthermore, we demonstrate that the two main QMP compounds 9-ODA and 9-HDA tested individually were as effective as the blend of all four major QMP compounds, suggesting considerable signal redundancy. Possible adaptive reasons for the observed complexity of the honeybee queen signal mix are discussed.

1. Introduction

Social insects are characterized by a division of labour in which the brood of fertile queens is cared for by largely sterile daughter workers. To maintain this reproductive division of labour, the queen emits specific pheromones, which induce the workers to remain sterile in her presence [1–5]. Originally, such queen pheromones were thought to be controlling agents that chemically suppress the workers' reproduction against their own reproductive interests [6–8]. More recent evidence, however, has largely discredited this 'queen control' hypothesis and instead interprets queen pheromones as honest signals for the presence of a fertile queen, to which the workers merely respond in their own best evolutionary interests [3,9–12]. Under this 'queen signal' hypothesis, queen pheromones induce worker sterility whenever the sociogenetic structure of the colony is such that the inclusive fitness costs of worker reproduction outweigh the potential direct fitness benefits [12–15]. For example, in many species, worker reproduction is made unprofitable by large costs to colony productivity [14–16] and/or by the fact that workers selectively detect and remove eggs laid by other workers—a process known as 'worker policing' [12,13,16–19]. In these cases, worker sterility ceases to be an evolutionary paradox, and instead ends up serving the workers' long-term genetic interests [3,12,20].

Although evidence suggests that most social insect lineages with specialized reproductive castes possess queen pheromones, few such pheromones have been identified to date. The first queen pheromones were identified in honeybees in the 1950s [21,22], and were found to be derived from the queen's mandibular

glands, containing a blend of five major compounds: the semi-volatile carboxylic acids (2*E*)-9-oxo-dec-2-enoic acid (9-ODA) and both enantiomers of (2*E*)-9-hydroxydec-2-enoic acid (9-HDA), along with two aromatics, methyl 4-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (homovanillyl alcohol, HVA) [23] (table 1). Subsequent research showed that these queen mandibular pheromones (QMP) serve diverse functions—not only inhibiting worker ovary activation [24,25] and queen cell production [26] but also mediating worker retinue formation around the queen [5,23,27] and acting as a sex attractant [28]. It took another 50 years, however, before experiments with synthetic pheromones succeeded in identifying queen pheromones in other lineages of social insects, including ants, wasps and bumblebees [2–4,29–34]. These studies showed that the queen pheromones in other social insects were from entirely different chemical classes than QMP constituents, consisting of long-chain hydrocarbons that were much more abundant in queens than in workers. Furthermore, queens of different species were often found to employ the same compounds as queen pheromones [2,3,32–35]. In particular, multiple species of ants, wasps and bumblebees were shown to share specific cuticular hydrocarbons as queen pheromones, including linear alkanes, 3-methylalkanes and alkenes [2,4]. Likewise, non-polar cuticular extracts from queens of a stingless bee species were shown to inhibit worker reproduction, and several linear and methyl-branched alkanes in the extracts induced electroantennographic responses from antennae of workers [36].

The important role of cuticular queen pheromones in ants, wasps and several species of corbiculate bees contrasts starkly with what is known of the honeybee, where most studies to date have focused on the queen's mandibular pheromones [1,5], despite comparative phylogenetic and empirical evidence suggesting that some cuticular compounds might also act as queen pheromones [3,37,38]. For example, queens from which the mandibular glands were removed were found to inhibit worker reproduction as effectively as intact queens [37,39,40], and extracts of the tergal glands, which produce specific esters, fatty acids and alkenes on the queen's dorsal cuticle [41,42], inhibit worker ovary development [43] and induce worker retinue behaviour [44]. However, it is still not known which classes of cuticular compounds actually induce honeybee sterility.

The aim of the present study, therefore, was to directly address this question by bioassaying defined blends of honeybee cuticular and tergal gland components and testing whether they suppressed the activation of the workers' ovaries. To this end, we first carried out a comprehensive gas chromatography–mass spectrometry (GC–MS) analysis of the cuticular profiles of *Apis mellifera* queens and workers. Subsequently, we bioassayed synthetic blends of queen-characteristic alkenes, esters and carboxylic acids to test the extent to which they inhibited worker ovary activation when compared with both the known QMP blend and each of the major QMP compounds individually. With the exception of 9-ODA [25,45,46] and 9-HDA [25], the extent to which the individual QMP compounds might suppress honeybee worker reproduction is unknown [3,4].

2. Material and methods

(a) Identification of queen-characteristic compounds

To shortlist putative honeybee queen pheromones, we first compared the cuticular profiles of European honeybee egg-laying queens, virgin queens and workers using GC–MS analysis, as no single published study has presented a comprehensive analysis of total cuticular extracts of female honeybee castes (but see [42] for data on alkene isomers specific to European honeybee queens and [47,48] for analyses of virgin honeybee queens). These data were then combined with previously published data on queen and worker profiles of African subspecies *A. mellifera scutellata* cuticular tergal glands [41], located underneath the dorsal part of the queen's cuticle. These glands were previously shown to release pheromones that stop workers from reproducing [43] and induce worker retinue behaviour [44]. Following the protocol used in Van Oystaeyen *et al*. [2], putative queen pheromones were shortlisted on the basis of the compounds' relative abundance (keeping only compounds with a relative peak area greater than 1%) and the extent to which they were characteristic for queens, which was quantified using the standardized difference in relative peak area in queens versus workers (Cohen's *d* values, cf. table 1). Subsequently, synthetic blends of these compounds grouped by structural class and source (cuticular or tergal gland) were tested in bioassays to examine the extent to which they inhibit worker ovary activation. Profiles of the abdominal Dufour's gland, which is also suspected to release queen or fertility signals [49–51], were not analysed separately, given that the compounds produced by this gland are also present on the cuticle, including most of the previously reported fertility-associated long-chain wax esters [49,50].

For the GC–MS analysis of the overall cuticular profiles of European mixed race honeybee castes, we first collected mated, egg-laying queens ($n = 11$) and workers ($n = 20$) in the spring and summer of 2014 from a randomly selected set of colonies. In addition, we sampled virgin queens ($n = 18$), which were reared from 1-day-old larvae derived from a random set of donor colonies. Individuals were then extracted in 1 ml of pentane (HPLC, Sigma-Aldrich) for 10 min, after which the samples were evaporated at room temperature to dryness. After resuspending these samples in 200 µl of hexane, we injected 2 µl of each sample into a Shimadzu QP2010Ultra coupled gas chromatograph/mass spectrometer, using a DB-5 ms capillary column (30 m × 0.25 mm × 0.25 µm) and a temperature programme as described in [38], with an initial temperature profile consisting of 1 min at 70°C, two temperature ramps from 70°C to 150°C at 20°C min^{-1} and from 150°C to 320°C at 3°C min^{-1}, after which the final temperature of 320°C was held for 15 min. Helium was used as a carrier gas at a flow rate of 1 ml min^{-1}, a splitless injection and an inlet temperature of 280°C, with a final pressure of 75 kPa. The ion source temperature was set to 300°C. Peaks in the total ion chromatogram were integrated using the Shimadzu GCMS Solutions software. A heptane to tetracontane alkane ladder external standard (0.01 mg ml^{-1}) was run to be able to infer cubic spline interpolated retention indices for all compounds (cf. [38], electronic supplementary material, table S1). Compounds were identified based on their mass spectra (expected fragmentation patterns and similarity to spectra in the NIST2014 mass spectral library) and retention indices (similarity to values given in the NIST2014 retention index library or in the literature). Alkene and alkadiene double bond positions were determined using DMDS derivatisation where possible [52]. Absolute amounts of the shortlisted queen pheromones that were present on a single egg-laying queen ('queen equivalents', table 1) were determined from measured peak areas, taking into account response factors relative to the closest eluting linear alkane of the pure synthetic compounds (measured from a separate set of $n = 20$ three-week-old, laying queens, table 1). Log_{10} transformed relative peak areas of all compounds were statistically compared among egg-laying queens, virgin queens and workers using robust linear model (*rlm*) analysis, using R's MASS package and *post hoc* tests coupled with *FDR* *p*-value adjustment to

Table 1. Previously known and newly identified honeybee queen pheromones tested in this study. Seven cuticular esters and four alkenes (rel. peak area queens greater than 1% and Cohen's *d* queens versus workers greater than 2.50) along with four tergal gland esters and three carboxylic acids (Cohen's $d > 0.77$) were shortlisted. Each of these sets of compounds was tested as synthetic blends in bioassays. The amount of each of the compounds present on a single queen (Qeq = one queen equivalent) was based on GC–MS analysis of 20 (cuticular) and four (tergal gland) honeybee queens. For comparison, we also compared bioactivity of our four synthetic blends of newly identified putative queen pheromones with that of the known queen mandibular pheromones (QMP major compound mix, plus each of the four major compounds tested individually, Qeqs based on data provided by Intko Supply Ltd).

compound class and origin	compounds	common name/abbreviation	Cohen's *d* Q versus W	rel. peak area in queens (%)	1 Qeq (μg)	supplier
newly identified queen pheromones						
cuticular esters	tetradecanoic acid, tetradecyl ester	myristyl myristate	4.12	1.56	1.18	Sigma-Aldrich
	(*Z*)-9-hexadecenoic acid, tetradecyl ester	myristyl palmitoleate	2.97	2.49	3.43	synthesized
	tetradecanoic acid, hexadecyl ester	palmityl myristate	5.87	3.26	1.45	Sigma-Aldrich
	(*Z*)-9-octadecenoic acid, tetradecyl ester	myristyl oleate	3.72	2.09	33.87	Molekula
	(*Z*)-9-hexadecenoic acid, hexadecyl ester	palmityl palmitoleate	4.43	2.45	4.48	synthesized
	hexadecanoic acid, hexadecyl ester	palmityl palmitate	4.72	1.01	0.92	Sigma-Aldrich
	(*Z*)-9-octadecenoic acid, hexadecyl ester	palmityl oleate	3.34	2.68	14.11	Molekula
cuticular alkenes	(*Z*)-15-tritriacontene	15-$C_{33:1}$	6.82	2.22	1.81	synthesized
	(*Z*)-15-pentatriacontene	15-$C_{35:1}$	5.99	2.28	1.75	synthesized
	(*Z*)-15-heptatriacontene	15-$C_{37:1}$	5.63	2.00	1.22	synthesized
	(*Z*)-15-nonatriacontene	15-$C_{39:1}$	4.12	1.37	1.03	synthesized
Tergal gland esters	hexadecanoic acid, methyl ester	methyl palmitate	3.62	4.40	0.25	Sigma-Aldrich
	(*Z*)-9-octadecenoic acid, methyl ester	methyl oleate	2.70	1.80	0.08	Sigma-Aldrich
	decanoic acid, decyl ester	capryl caprate	1.16	1.30	0.06	TCI Europe N.V.
	dodecanoic acid, decyl ester	decyl laurate	0.83	0.90	0.04	Molekula
tergal gland acids	hexadecanoic acid	palmitic acid	0.85	8.00	8.67	Sigma-Aldrich
	(Z)-9-octadecenoic acid	oleic acid	3.15	47.20	44.95	Sigma-Aldrich
	octadecanoic acid	stearic acid	0.77	12.30	12.32	Sigma-Aldrich
known queen pheromones						
queen mandibular pheromones	QMP major compound blend	QMP	—	—	422	Intko Supply Ltd
	(*E*)-9-oxodec-2-enoic acid	9-ODA	—	—	250	Intko Supply Ltd
	(2*E*,9*S*) & (2*E*,9*R*)-9-hydroxy-2-decenoic acid	9-HDA	—	—	150	Intko Supply Ltd
	methyl 4-hydroxybenzoate	HOB	—	—	20	Sigma-Aldrich
	4-hydroxy-3-methyoxyphenylethanol	homovanillyl alcohol (HVA)	—	—	2	Sigma-Aldrich

correct for multiple testing (electronic supplementary material, table S1). A heatmap of the relative differences in the profiles was produced using the R *pheatmap* package combined with UPGMA hierarchical row and column clustering, using one minus the Pearson correlation as distance metric (figure 1). In order to make the scaling of the different compounds comparable in the heatmap, we used row *z*-scores (standardized differences compared to the row average) calculated on $\log_{10}$ transformed relative peak areas.

To shortlist possible bioactive queen pheromones derived from the tergal glands we focused our attention on the queen–worker differences reported in *A. mellifera scutellata* by Wossler & Crewe [41], and retained the top seven most queen characteristic compounds based on the Cohen's *d* effect size [2] (table 1). Absolute quantities of the shortlisted compounds present in the tergal glands were based on the relative peak areas reported by Wossler & Crewe [41], using the measured amount of stearic acid present in extracts of the tergal glands of European honeybee queens as a baseline, as this was the most prominent acid in our samples, and taking into account measured response factors for all the compounds. To quantify the amount of stearic acid present in single queens, we dissected the tergites of four queens obtained from our hives in July 2018. The tergites were ground in 250 µl of hexane and the remaining pieces were taken out after 40 min. Subsequently, the solvent was evaporated to dryness, 100 ml of hexane was added after which the solution was vortexed for 15 s and 1 ml was injected into the GC–MS.

Finally, a blend of the major QMP compounds and each of the individual major compounds (9-ODA, two isomers of 9-HDA, HOB and HVA) were included as treatments and the typical amount present in single queens was provided by the supplier (Intko Supply Ltd, Vancouver, Canada) (table 1).

(b) Bioassays to identify novel honeybee queen pheromones

To determine the bioactivity of the shortlisted cuticular and tergal gland queen pheromones, we performed bioassays to test the extent to which synthetic versions of each class of compounds inhibited worker ovary activation. For the different treatments, the cuticular and tergal gland compounds were grouped by structural class and source into different mixes in acetone solvent: (1) cuticular esters, (2) cuticular alkenes, (3) tergal gland esters and (4) tergal gland acids. Each mix contained the different compounds in the ratios and amounts in which they occurred naturally in honeybee queens, listed in table 1. We also included a negative acetone solvent control plus QMP as a positive control, as well as its major individual components, resulting in a total of 10 treatments (table 1). Our hypothesis was that administering a bioactive queen pheromone would mimic the presence of a live queen and induce worker ovary inhibition. Among the shortlisted putative queen pheromones, most could be commercially obtained in synthetic form (table 1). Myristyl palmitoleate, palmityl palmitoleate and four queen-specific N-15 alkenes [42], were not commercially available and hence were synthesized (described in the electronic supplementary material).

For the bioassays, performed in July 2018, we pipetted a daily dose of 0.5 queen equivalents of the synthetic pheromone compounds (table 1) in 150 µl of acetone onto a microscope slide that was put on top of an empty piece of honeybee comb placed upright inside a 17 × 7 × 12 cm wooden box. Control slides were treated only with acetone. The acetone was allowed to evaporate before placing the slide into the nest box. The boxes each contained a random mix of 50 newly emerged worker bees, that had emerged in an incubator overnight, derived from four European mixed race source colonies housed in our apiary in Leuven. The boxes were set up in a climate-controlled room (28°C and 14 L : 10 D day–night cycle) with a glass wall on one side and a mesh wall on the other. Inside the boxes, the workers were provided ad libitum with water, sugar water (50 : 50), and freshly collected beebread. After three weeks of treatments, the bees were frozen at −20°C before the workers were dissected under a Leica MSV266 stereo microscope to assess their ovary development. The ovary stages were scored according to the scale of Velthuis [53], and as in [53], ovaries of classes 2 or 3 were considered as developed. Each treatment was replicated five times, and each replicate of 10 treatments was set up in blocks on successive days. The significance of the treatments on the proportion of workers with developed ovaries was determined using a binomial generalized linear mixed model (GLMM) using R package *lme4* v.1.1 [54]. In this model, 'replicate' and 'nest box' were added as random intercepts. *Post hoc* tests against the negative control were performed using the *emmeans* package v.1.3.1. Because all treatments were *a priori* expected to inhibit worker ovary development and none of the queen pheromone treatments described in literature ever yielded significant enhancement of worker ovary development [4], one-sided *p*-values were used for these tests, but all *p*-values were *FDR* adjusted to correct for multiple testing. The synergistic effects between 9-ODA and 9-HDA were tested using the same model and adding the interaction factor.

3. Results

(a) Identification of queen-characteristic cuticular compounds

GC–MS analysis of total cuticular extracts of European honeybee egg-laying queens, virgin queens, and workers resulted in the identification of a total of 120 compounds, of which 105 were hydrocarbons (25 linear alkanes, 21 methylalkanes, 27 dimethylalkanes, 29 alkenes and three alkadienes), 11 were wax esters, two alcohols, one was an aldehyde and one a terpenoid. Significantly, each class of individuals featured characteristic sets of compounds (figure 1; electronic supplementary material, table S1), with 22 and 26 compounds each being significantly and more than twice as abundant on the cuticle of egg-laying queens and virgin queens respectively, when compared with workers (more than a twofold difference in relative peak area and *FDR* corrected p-value < 0.05, cf. electronic supplementary material, table S1). Wax esters and long-chain linear alkenes with chain lengths between 29 and 39 carbons were most characteristic of egg-laying queens. By contrast, some medium-length linear alkanes, monomethyl-branched long-chain alkanes and shorter-chain alkenes were most characteristic of virgin queens (see electronic supplementary material, table S1 for *FDR* corrected *p*-values). Based on these results, we shortlisted seven wax esters and four alkenes as putative queen pheromones, as they each had an average relative peak area in queens greater than 1% and a standardized difference in relative peak abundance in queens versus workers (Cohen's *d*) greater than 2.5 (cf. [2], table 1). In addition, we also selected four tergal gland wax esters and three carboxylic acids as being putative honeybee queen pheromones, based on earlier data from African *A. mellifera scutellata* bees [41] (Cohen's $d > 0.77$) (cf. Material and methods).

Interestingly, some of the compounds that we shortlisted were previously suggested to be caste- or fertility-linked, including a series of queen-specific alkene isomers with a double bond in the N-15 position first reported from honeybee tergal gland extracts [42] (compounds included in our 'cuticular alkenes' mix in table 1) and a series of wax esters reported in honeybee queen Dufour's glands [49,50,55] (all compounds included in our 'cuticular esters' mix).

Figure 1. Queen-characteristic cuticular compounds in honeybees identified based on GC–MS analysis. Using *z*-scores calculated on the $\log_{10}$ transformed relative peak areas of each compound, the heatmap shows that compounds clearly cluster in three groups characteristic for workers, egg-laying queens and virgin queens, respectively. The compounds highlighted in blue and purple are esters and alkenes that showed the largest differences between queens and workers (Cohen's $d > 2.5$ and relative peak area in queens greater than 1%, cf. table 1 and electronic supplementary material, table S1 and figure S1) and were tested as synthetic blends for queen pheromone bioactivity in our bioassays. (Online version in colour.)

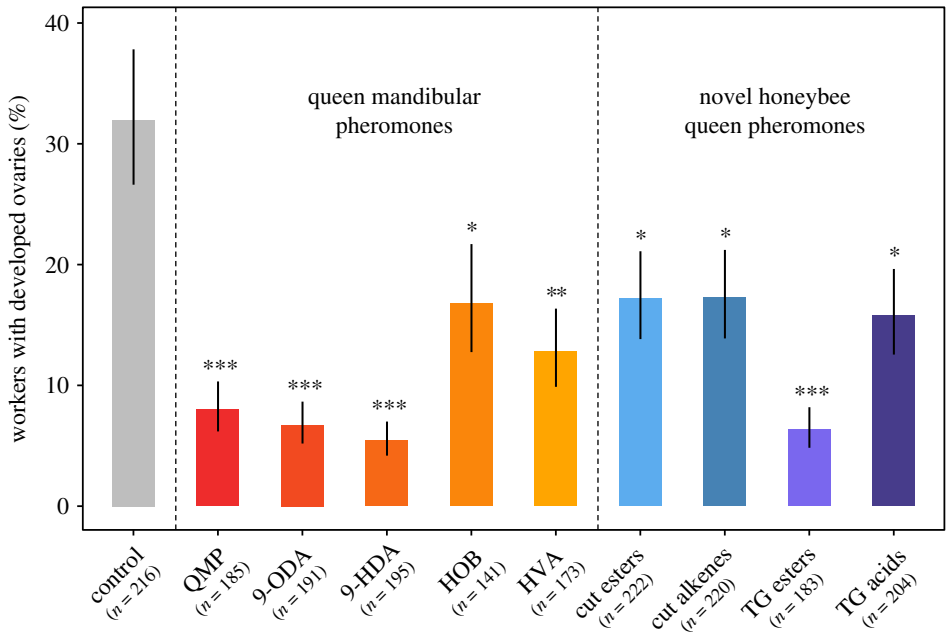

Figure 2. Bioassays reveal a large panel of novel bioactive honeybee queen pheromones. The synthetic blends of queen-characteristic cuticular esters and alkenes (cf. Figure 1; electronic supplementary material, figure S1) as well as tergal gland esters and carboxylic acids (table 1) all significantly inhibit worker ovary development in queenless worker groups compared to the solvent-only control. The tergal gland compounds were as effective at inhibiting worker ovary development as the total queen mandibular pheromone mix QMP and its two major constituents 9-ODA and 9-HDA, while the cuticular esters, cuticular alkenes and tergal gland acids were as effective as the two other major components of the total QMP mix, HOB and HVA. The columns and whiskers show the marginal predicted means of a binomial GLMM plus the 95% confidence bounds. Significances are indicated by asterisks (n = total number of workers dissected across each of the five replicate groups per treatment, see electronic supplementary material, table S2 for details). (Online version in colour.)

(b) Bioassays to identify novel honeybee queen pheromones

All synthetic blends of putative queen pheromones that we tested proved to significantly inhibit worker ovary activation relative to the solvent-only control (figure 2). The tergal gland ester mix had the strongest effect (binomial GLMM, $p = 0.00008$) and inhibited worker ovary development to a similar extent as the known QMP blend and its two major components, 9-ODA and 9-HDA, reducing the proportion of workers with developed ovaries from 32.0% in the solvent control to 6.3% in the tergal gland ester treatment (figure 2), a sevenfold reduction in ovary activation when compared with the control (electronic supplementary material, table S2). The cuticular esters, cuticular alkenes and tergal gland acids inhibited worker ovary development to a more limited extent, resulting in proportions of workers with developed ovaries of 17.1%, 17.1% and 15.8%, respectively, reflecting *ca* twofold decreases in ovary activation when compared with the control (all $p < 0.05$, figure 2; electronic supplementary material, table S2).

The two major constituents of the QMP, 9-ODA and 9-HDA, when administered individually, inhibited worker ovary development to a similar extent as a blend of all four major QMP components, with 6.7% and 5.4% of the workers having developed ovaries in the 9-ODA and 9-HDA treatments respectively versus 8.0% in the QMP treatment (figure 2). Although each of these treatments significantly inhibited worker ovary development compared to the control (all $p < 0.0001$, electronic supplementary material, table S2), there were no significant differences in the effectiveness of the individual components compared to the full blend of QMP components. This suggests that there is no synergy among the individual components, but rather that there is considerable signal redundancy. The levels of worker ovary inhibition induced by HVA and HOB, which until now had not been tested individually [3,4], were analogous to those observed for the cuticular ester, cuticular alkene and tergal gland acid queen pheromone blends (HVA: $p = 0.008$, odds ratio = 2.3; HOB: $p = 0.035$, odds ratio = 3.2, figure 2; electronic supplementary material, table S2).

4. Discussion

Overall, our results demonstrate that honeybee pheromones inducing worker sterility are not restricted to the widely studied QMP [1,5], but also include a variety of cuticular and tergal gland compounds. Specifically, synthetic mixes of queen-characteristic wax esters, linear alkenes and carboxylic acids (table 1) all inhibited worker ovary development in bioassays (figure 2). This provides experimental support that low volatility cuticular compounds also act as honeybee queen pheromones, as previously hypothesized [3,4,37,38,42,43,56,57]. In addition, our study is the first to determine the extent to which the individual major QMP compounds inhibit worker reproduction in European honeybees [4]. Surprisingly, we found no evidence for synergy among the QMP compounds in suppressing worker ovary activation, with 9-ODA and 9-HDA being as active as the full blend (figure 2). Likewise, the queen tergal gland esters inhibited worker ovary development as strongly as the full QMP mix (figure 2). These results show that there is substantial functional redundancy in the pheromones involved in queen signalling, with several individual compounds or blends being equally effective in inhibiting worker ovary development [37]. This seems to contrast with the positive synergistic effects reported for the pheromones that induce retinue behaviour [23,27]. On

the other hand, we did also find differing effectiveness between some of the blends and QMP compounds and together with the fact that we did not test all the queen-characteristic compounds individually, this also points to the likely presence of queen pheromone compounds that act additively or synergistically.

Several studies had already suggested that additional pheromones besides QMP could play a role in inducing worker sterility in honeybees, but none succeeded in identifying the bioactive compounds or compound classes [37,39,40,43,50,57,58]. For example, it was shown that queens from which the mandibular glands were removed inhibited worker reproduction as effectively as intact queens [37,39,40]. Our experiments showed that queen-specific cuticular alkenes and wax esters as well as tergal gland acids and esters all induced significant worker ovary inhibition (figure 2; electronic supplementary material, table S2).

For cuticular alkenes, a likely function as queen pheromone components was suggested by a phylogenetic analysis of queen-characteristic compounds across the clade of corbiculate bees and their direct ancestors [38], and—more generally—was expected from the proof for hydrocarbons playing a conserved role as queen and fertility signals across other clades of social insects [2–4,29–34,59–61]. Our cuticular ester blend coincides with compounds reported from the queen's Dufour's gland, and their observed bioactivity is in line with earlier inconclusive evidence for them acting as a worker sterility-inducing queen signal [50,57,58]. In addition, earlier results showed that these queen's Dufour's gland esters also induce worker retinue behaviour [50]. Finally, for the tergal gland compounds, our bioassays are in agreement with tergal gland extracts inhibiting worker ovary development and inducing worker retinue behaviour in African honeybees [43,44], and further establish that the esters most strongly inhibited worker reproduction, even though the tergal gland acids were also bioactive (figure 1). Interestingly, the tergal gland ester mix also included two compounds—methyl palmitate and methyl oleate—that have been reported to be part of the sterility-inducing brood pheromone esters [62–65]. These brood pheromones encourage the workers to care for the brood and signal that they should rear female larvae into replacement queens before starting to reproduce themselves.

Altogether, our results show that honeybees have evolved a many-layered system to maintain an effective reproductive division of labour, with the multicomponent queen pheromone blend likely comprising both redundant and synergistically acting signalling molecules. This raises the question what the selective advantages were for honeybees to develop such a complex signalling system. Several hypotheses have been suggested. For example, multiple redundant 'backup' signals could make the queen signal more effective and reliable, and would both ensure that the message was perceived and correct for possible errors of signal detection and identification, which are likely given the complex chemical background in which the pheromonal signals are perceived [59,66]. It could also be adaptive to produce multiple non-redundant queen signals in the form of the different QMP, tergal gland and Dufour's gland compounds that synergistically induce retinue formation [23,27,44,50], because the combination of compounds could increase the information content of the signal [66] or cause it to act over broader spatial scales due to the differing volatility of the components [23,27,44,50]. Likewise, a complex queen pheromone bouquet could be required for workers to correctly infer the colony's reproductive state, which is partly signalled by the queen (reviewed in [67]). For example, in the swarming season, only the combination of both queen mandibular gland and tarsal gland pheromones could decrease queen cell rearing, whereas neither pheromone by itself elicited this effect [68]. Hence, the many purposes of queen pheromones in a honeybee colony might explain their observed complexity.

Another set of hypotheses have proposed that the complexity of the honeybee queen pheromone is linked with queen–worker arms races over reproduction. The original idea was formulated in the context of the 'queen control' hypothesis, and was suggested to be the result of the queen releasing chemicals that manipulate the workers to remain sterile, which would induce workers to evolve tolerance to these chemicals, after which the queen in turn would evolve novel compounds as a counter-defence to remain in control [3,58,69]. This idea is disproved here since all individual QMP components were bioactive queen pheromones. The fact that the QMP component HVA appears to directly bind to dopamine receptors in the brain of honeybee workers to induce worker sterility, thereby bypassing the sensory systems, is sometimes cited in support of the 'queen control' hypothesis [69–71]. Nevertheless, from the perspective of the 'queen signal' hypothesis, this could equally be interpreted as the queen signals merely having evolved through the exploitation of a pre-existing physiological bias, which is one of the common routes by which signals evolve in general [72].

Another way in which arms races could play a role in the evolution of honeybee queen signals is based on the idea that individual workers could benefit from mimicking the queen's signals, thus enabling them to successfully reproduce [19]. A redundant queen signal would facilitate such cheating, as mimicking any one of the individual compounds would be sufficient to achieve an almost full effect, whereas additively or synergistically acting signals would be more resistant to such cheating. In honeybee colonies, workers attempting reproduction are aggressed by other workers and their eggs are destroyed [17,19]. If rare workers inside the colony are able to mimic the queen's signals and become insensitive to them, this would allow them to activate their ovaries without interference and lay eggs that can evade policing [19]. Multiple lines of evidence suggest that individual workers can indeed cheat by mimicking one or several queen pheromone compounds, including several that were confirmed to be bioactive in our study [73–79].

Our results show that honeybee queen pheromones are complex, and comprise several distinct signalling compounds that might work in synergy but also appear to be partly redundant [37]. Further comparative studies in which a greater variety of compounds and compound classes are tested for queen pheromone activity may be desirable to test how queen pheromone signal complexity evolved across a wider variety of clades of social insects.

Data accessibility. All datasets from this study and R scripts that were used to analyse the data are available via the Dryad Repository https://doi.org/10.5061/dryad.s31v04d. [80]

Authors' contributions. S.A.P. participated in the design of the study, carried out the bioassays, participated in data analysis and chemical analyses, and drafted the manuscript; R.C.O. participated in the design of the study, carried out chemical analyses and critically revised the manuscript, U.R.E. collected field data and critically revised the manuscript; J.G.M. synthesized the chemical compounds and critically revised both the chemical analyses and the manuscript; J.S.Z. collected field data, participated in the design of the study and carried out chemical analyses; T.W. conceived of the study, designed the study, carried out the statistical analyses and helped draft the

manuscript. All authors gave final approval for publication and agree to be held accountable for the work performed therein.

Competing interests. We declare we have no competing interests.

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References

1. Le Conte Y, Hefetz A. 2008 Primer pheromones in social hymenoptera. *Annu. Rev. Entomol.* **53**, 523–542. (doi:10.1146/annurev.ento.52.110405.091434)
2. Van Oystaeyen A *et al.* 2014 Conserved class of queen pheromones stops social insect workers from reproducing. *Science* **343**, 287–290. (doi:10.1126/science.1244899)
3. Oi CA, van Zweden JS, Oliveira RC, Van Oystaeyen A, Nascimento FS, Wenseleers T. 2015 The origin and evolution of social insect queen pheromones: novel hypotheses and outstanding problems. *Bioessays* **37**, 808–821. (doi:10.1002/bies.201400180)
4. Holman L. 2018 Queen pheromones and reproductive division of labor: a meta-analysis. *Behav. Ecol.* **29**, 1199–1209. (doi:10.1093/beheco/ary023)
5. Slessor KN, Winston ML, Le Conte Y. 2005 Pheromone communication in the honeybee (*Apis mellifera* L.) *J. Chem. Ecol.* **31**, 2731–2745. (doi:10.1007/s10886-005-7623-9)
6. Fletcher DJC, Ross KG. 1985 Regulation of reproduction in eusocial Hymenoptera. *Annu. Rev. Entomol.* **30**, 319–343. (doi:10.1146/annurev.en.30.010185.001535)
7. Hölldobler B, Wilson EO. 1990 *The ants*, 746 p. Berlin, Germany: Springer.
8. Strauss K, Scharpenberg H, Crewe RM, Glahn F, Foth H, Moritz RFA. 2008 The role of the queen mandibular gland pheromone in honeybees (*Apis mellifera*): honest signal or suppressive agent? *Behav. Ecol. Sociobiol.* **62**, 1523–1531. (doi:10.1007/s00265-008-0581-9)
9. Keller L, Nonacs P. 1993 The role of queen pheromones in social insects: queen control or queen signal? *Anim. Behav.* **45**, 787–794. (doi:10.1006/anbe.1993.1092)
10. Holman L. 2012 Costs and constraints conspire to produce honest signaling: insights from an ant queen pheromone. *Evolution* **66**, 2094–2105. (doi:10.1111/j.1558-5646.2012.01603.x)
11. Peso M, Elgar MA, Barron AB. 2014 Pheromonal control: reconciling physiological mechanism with signalling theory. *Biol. Rev.* **90**, 542–559. (doi:10.1111/brv.12123)
12. van Zweden JS, Bonckaert W, Wenseleers T, d'Ettorre P. 2014 Queen signaling in social wasps. *Evolution* **68**, 976–986. (doi:10.1111/evo.12314)
13. Wenseleers T, Hart AG, Ratnieks FLW. 2004 When resistance is useless: policing and the evolution of reproductive acquiescence in insect societies. *Am. Nat.* **164**, E154–E167. (doi:10.1086/425223)
14. Wenseleers T, Ratnieks FLW. 2006 Comparative analysis of worker reproduction and policing in eusocial Hymenoptera supports relatedness theory. *Am. Nat.* **168**, E163–E179. (doi:10.1086/508619)
15. Wenseleers T, Helanterä H, Alves DA, Dueñez-Guzmán E, Pamilo P. 2013 Towards greater realism in inclusive fitness models: the case of worker reproduction in insect societies. *Biol. Lett.* **9**, 20130334. (doi:10.1098/rsbl.2013.0334)
16. Wenseleers T, Helanterä H, Hart A, Ratnieks FLW. 2004 Worker reproduction and policing in insect societies: an ESS analysis. *J. Evol. Biol.* **17**, 1035–1047. (doi:10.1111/j.1420-9101.2004.00751.x)
17. Ratnieks FLW, Foster KR, Wenseleers T. 2006 Conflict resolution in insect societies. *Annu. Rev. Entomol.* **51**, 581–608. (doi:10.1146/annurev.ento.51.110104.151003)
18. Wenseleers T, Ratnieks FL. 2006 Enforced altruism in insect societies. *Nature* **444**, 50. (doi:10.1038/444050a)
19. Ratnieks FLW, Wenseleers T. 2008 Altruism in insect societies and beyond: voluntary or enforced? *Trends Ecol. Evol.* **23**, 45–52. (doi:10.1016/j.tree.2007.09.013)
20. Ratnieks FLW, Foster KR, Wenseleers T. 2011 Darwin's special difficulty: the evolution of 'neuter insects' and current theory. *Behav. Ecol. Sociobiol.* **65**, 481–492. (doi:10.1007/s00265-010-1124-8)
21. Butler CG. 1957 The control of ovary development in worker honeybees (*Apis mellifera*). *Experientia* **6**, 256–257. (doi:10.1007/bf02157449)
22. Butler CG. 1959 The source of the substance produced by a queen honeybee (*Apis mellifera* L.) which inhibits development of the ovaries of the workers of her colony. *Proc. R. Entomol. Soc. Lond. (A)* **34**, 137–138. (doi:10.1111/j.1365-3032.1959.tb00249.x)
23. Slessor KN, Kaminski L-A, King GGS, Borden JH, Winston ML. 1988 Semiochemical basis of the retinue response to queen honey bees. *Nature* **332**, 354–356. (doi:10.1038/332354a0)
24. Hoover SE, Keeling CI, Winston ML, Slessor KN. 2003 The effect of queen pheromones on worker honey bee ovary development. *Naturwissenschaften* **90**, 477–480. (doi:10.1007/s00114-003-0462-z)
25. Tan K, Liu X, Dong S, Wang C, Oldroyd BP. 2015 Pheromones affecting ovary activation and ovariole loss in the Asian honey bee *Apis cerana*. *J. Insect. Physiol.* **74**, 25–29. (doi:10.1016/j.jinsphys.2015.01.006)
26. Melathopoulos AP, Winston ML, Pettis JS, Pankiw T. 1996 Effect of queen mandibular pheromone on initiation and maintenance of queen cells in the honey bee (*Apis mellifera* L.). *Can. Entomol.* **128**, 263–272. (doi:10.4039/Ent128263-2)
27. Keeling CI, Slessor KN, Higo HA, Winston ML. 2003 New components of the honey bee (*Apis mellifera* L.) queen retinue pheromone. *Proc. Natl Acad. Sci. USA* **100**, 4486–4491. (doi:10.1073/pnas.0836984100)
28. Gary NE. 1962 Chemical mating attractants in the queen honey bee. *Science* **136**, 773–774. (doi:10.1126/science.136.3518.773)
29. Smith AA, Hölldobler B, Liebig J. 2009 Cuticular hydrocarbons reliably identify cheaters and allow enforcement of altruism in a social insect. *Curr. Biol.* **19**, 78–81. (doi:10.1016/j.cub.2008.11.059)
30. Holman L, Jørgensen CG, Nielsen J, d'Ettorre P. 2010 Identification of an ant queen pheromone regulating worker sterility. *Proc. R. Soc. B* **277**, 3793–3800. (doi:10.1098/rspb.2010.0984)
31. Holman L. 2014 Bumblebee size polymorphism and worker response to queen pheromone. *PeerJ* **2**, e604. (doi:10.7717/peerj.604)
32. Holman L, Hanley B, Millar JG. 2016 Highly specific responses to queen pheromone in three *Lasius* ant species. *Behav. Ecol. Sociobiol.* **70**, 387–392. (doi:10.1007/s00265-016-2058-6)
33. Holman L, Lanfear R, d'Ettorre P. 2013 The evolution of queen pheromones in the ant genus *Lasius*. *J. Evol. Biol.* **26**, 1549–1558. (doi:10.1111/jeb.12162)
34. Oi CA, Millar JG, van Zweden JS, Wenseleers T. 2016 Conservation of queen pheromones across two species of vespine wasps. *J. Chem. Ecol.* **42**, 1175–1180. (doi:10.1007/s10886-016-0777-9)
35. Holman L, van Zweden JS, Oliveira RC, van Oystaeyen A, Wenseleers T. 2017 Conserved queen pheromones in bumblebees: a reply to Amsalem *et al*. *PeerJ* **5**, e3332. (doi:10.7717/peerj.3332)
36. Nunes TM *et al.* 2014 Queen signals in a stingless bee: suppression of worker ovary activation and spatial distribution of active compounds. *Sci. Rep.* **4**, 7449. (doi:10.1038/srep07449)
37. Maisonnasse A, Alaux C, Beslay D, Crauser D, Gines C, Plettner E, Le Conte Y. 2010 New insights into honey bee (*Apis mellifera*) pheromone communication. Is the queen mandibular pheromone alone in colony regulation? *Front. Zool.* **7**, 18. (doi:10.1186/1742-9994-7-18)
38. Oliveira RC, Oi CA, do Nascimento MMC, Vollet-Neto A, Alves DA, Campos MC, Nascimento F, Wenseleers T. 2015 The origin and evolution of queen and fertility signals in corbiculate bees. *BMC Evol. Biol.* **15**, 254. (doi:10.1186/s12862-015-0509-8)

39. Velthuis HHW. 1970 Queen substances from the abdomen of the honey bee queen. *J. Comp. Physiol. A* **70**, 210–221. (doi:10.1007/bf00297717)
40. Velthuis HHW, van Es J. 1964 Some functional aspects of the mandibular glands of the queen honeybee. *J. Apic. Res.* **3**, 11–16. (doi:10.1080/00218839.1964.11100076)
41. Wossler TC, Crewe RM. 1999 Mass spectral identification of the tergal gland secretions of female castes of two African honey bee races (*Apis mellifera*). *J. Apic. Res.* **38**, 137–148. (doi:10.1080/00218839.1999.11101004)
42. Smith R-K, Taylor OR. 1990 Unsaturated extracted hydrocarbon caste differences between European queen and worker honey bees, *Apis mellifera* L. (Hymenoptera: Apidae). *J. Kans Entomol. Soc.* **63**, 369–374. (doi:10.2307/25085192)
43. Wossler TC, Crewe RM. 1999 Honeybee queen tergal gland secretion affects ovarian development in caged workers. *Apidologie* **30**, 311–320. (doi:10.1051/apido:19990407)
44. Wossler TC, Crewe RM. 1999 The releaser effects of the tergal gland secretion of queen honeybees (*Apis mellifera*). *J. Insect. Behav.* **12**, 343–351. (doi:10.1023/a:1020839505622)
45. Butler CG, Fairey EM. 1963 The role of the queen in preventing oogenesis in worker honeybees. *J. Apic. Res.* **2**, 14–18. (doi:10.1080/00218839.1963.11100051)
46. Kaatz H-H, Hildebrandt H, Engels W. 1992 Primer effect of queen pheromone on juvenile hormone biosynthesis in adult worker honey bees. *J. Comp. Physiol. B* **162**, 1–5. (doi:10.1007/bf00296638)
47. Espelie KE, Butz VM, Dietz A. 1990 Decyl decanoate: a major component of the tergite glands of honeybee queens (*Apis mellifera* L.). *J. Apic. Res.* **29**, 15–19. (doi:10.1080/00218839.1990.11101192)
48. Babis M, Holman L, Fenske R, Thomas M, Baer B. 2014 Cuticular lipids correlate with age and insemination status in queen honeybees. *Insectes Soc.* **61**, 337–345. (doi:10.1007/s00040-014-0358-2)
49. Katzav-Gozansky T, Soroker V, Hefetz A, Cojocaru M, Erdmann DH, Francke W. 1997 Plasticity of caste-specific Dufour's gland secretion in the honey bee (*Apis mellifera* L.). *Naturwissenschaften* **84**, 238–241. (doi:10.1007/s001140050386)
50. Katzav-Gozansky T, Soroker V, Ibarra F, Francke W, Hefetz A. 2001 Dufour's gland secretion of the queen honeybee (*Apis mellifera*): an egg discriminator pheromone or a queen signal? *Behav. Ecol. Sociobiol.* **51**, 76–86. (doi:10.1007/s002650100406)
51. Katzav-Gozansky T, Soroker V, Hefetz A. 2002 Honeybees Dufour's gland—idiosyncrasy of a new queen signal. *Apidologie* **33**, 525–537. (doi:10.1051/apido:2002035)
52. Carlson DA, Roan CS, Yost RA, Hector J. 1989 Dimethyl disulfide derivatives of long chain alkenes, alkadienes, and alkatrienes for gas chromatography/mass spectrometry. *Anal. Chem.* **61**, 1564–1571. (doi:10.1021/ac00189a019)
53. Velthuis HHW. 1970 Ovarian development in *Apis mellifera* worker bees. *Entomol. Exp. Appl.* **13**, 377–394. (doi:10.1111/j.1570-7458.1970.tb00122.x)
54. Bates D, Maechler M, Bolker B, Walker S. 2014 lme4: Linear mixed-effects models using Eigen and S4. R package version 1, 1–23.
55. Katzav-Gozansky T, Hefetz A, Soroker V. 2007 Brain modulation of Dufour's gland ester biosynthesis *in vitro* in the honeybee (*Apis mellifera*). *Naturwissenschaften* **94**, 407–411. (doi:10.1007/s00114-006-0206-y)
56. Katzav-Gozansky T, Boulay R, Soroker V, Hefetz A. 2004 Queen-signal modulation of worker pheromonal composition in honeybees. *Proc. R. Soc. Lond. B* **271**, 2065–2069. (doi:10.1098/rspb.2004.2839)
57. Katzav-Gozansky T, Boulay R, Soroker V, Hefetz A. 2006 Queen pheromones affecting the production of queen-like secretion in workers. *J. Comp. Physiol. A* **192**, 737–742. (doi:10.1007/s00359-006-0110-0)
58. Hefetz A, Katzav-Gozansky T. 2004 Are multiple honeybee queen pheromones indicators for a queen–workers arms race? *Apiacta* **39**, 44–52.
59. Smith AA, Liebig J. 2017 The evolution of cuticular fertility signals in eusocial insects. *Curr. Opin. Insect. Sci.* **22**, 79–84. (doi:10.1016/j.cois.2017.05.017)
60. Monnin T. 2006 Chemical recognition of reproductive status in social insects. *Ann. Zool. Fenn.* **43**, 515–530.
61. Liebig J. 2010 Hydrocarbon profiles indicate fertility and dominance status in ant, bee, and wasp colonies. In *Insect hydrocarbons: biology, biochemistry, and chemical ecology* (eds GJ Blomquist, AG Bagnères), pp. 282–324. Cambridge, UK: Cambridge University Press.
62. Le Conte Y, Arnold G, Trouiller J, Masson C, Chappe B. 1990 Identification of a brood pheromone in honeybees. *Naturwissenschaften* **77**, 334–336. (doi:10.1007/bf01138390)
63. Arnold G, Le Conte Y, Trouiller J, Hervet H, Chappe B, Masson C. 1994 Inhibition of worker honeybee ovaries development by a mixture of fatty acid esters from larvae. *C R Acad. Sci. Ser. III Sci. Vie* **317**, 511–515.
64. Mohammedi A, Paris A, Crauser D, Le Conte Y. 1998 Effect of aliphatic esters on ovary development of queenless bees (*Apis mellifera* L.). *Naturwissenschaften* **85**, 455–458. (doi:10.1007/s001140050531)
65. Pankiw T, Garza C. 2007 Africanized and European honey bee worker ovarian follicle development response to racial brood pheromone extracts. *Apidologie* **38**, 156–163. (doi:10.1051/apido:2006066)
66. Partan SR, Marler P. 2005 Issues in the classification of multimodal communication signals. *Am. Nat.* **166**, 231–245. (doi:10.1086/431246)
67. Grozinger CM, Richards J, Mattila HR. 2014 From molecules to societies: mechanisms regulating swarming behavior in honey bees (*Apis* spp.). *Apidologie* **45**, 327–346. (doi:10.1007/s13592-013-0253-2)
68. Lensky Y, Slabezki Y. 1981 The inhibiting effect of the queen bee (*Apis mellifera* L.) foot-print pheromone on the construction of swarming queen cups. *J. Insect. Physiol.* **27**, 313–323. (doi:10.1016/0022-1910(81)90077-9)
69. Kocher SD, Grozinger CM. 2011 Cooperation, conflict, and the evolution of queen pheromones. *J. Chem. Ecol.* **37**, 1263–1275. (doi:10.1007/s10886-011-0036-z)
70. Beggs KT, Glendining KA, Marechal NM, Vergoz V, Nakamura I, Slessor KN, Mercer AR. 2007 Queen pheromone modulates brain dopamine function in worker honey bees. *Proc. Natl. Acad. Sci. USA* **104**, 2460–2464. (doi:10.1073/pnas.0608224104)
71. Beggs KT, Mercer AR. 2009 Dopamine receptor activation by honey bee queen pheromone. *Curr. Biol.* **19**, 1206–1209. (doi:10.1016/j.cub.2009.05.051)
72. Stokl J, Steiger S. 2017 Evolutionary origin of insect pheromones. *Curr. Opin. Insect. Sci.* **24**, 36–42. (doi:10.1016/j.cois.2017.09.004)
73. Niu D-F, Pirk CW, Zheng H-Q, Ping S, Shi J-H, Cao L-F, Hu F-L. 2016 Reproductive traits and mandibular gland pheromone of anarchistic honey bee workers *Apis mellifera* occurring in China. *Apidologie* **47**, 515–526. (doi:10.1007/s13592-015-0396-4)
74. Martin SJ, Chaline N, Oldroyd BP, Jones GR, Ratnieks FLW. 2004 Egg marking pheromones of anarchistic worker honeybees (*Apis mellifera*). *Behav. Ecol.* **15**, 839–844. (doi:10.1093/beheco/arh089)
75. Sole CL, Kryger P, Hefetz A, Katzav-Gozansky T, Crewe RM. 2002 Mimicry of queen Dufour's gland secretions by workers of *Apis mellifera scutellata* and *A. m. capensis*. *Naturwissenschaften* **89**, 561–564. (doi:10.1007/s00114-002-0370-7)
76. Schäfer M, Dietemann V, Pirk C, Neumann P, Crewe R, Hepburn H, Tautz J, Crailsheim K. 2006 Individual versus social pathway to honeybee worker reproduction (*Apis mellifera*): pollen or jelly as protein source for oogenesis? *J. Comp. Physiol. A* **192**, 761. (doi:10.1007/s00359-006-0112-y)
77. Okosun OO, Pirk CWW, Crewe RM, Yusuf AA. 2017 Glandular sources of pheromones used to control host workers (*Apis mellifera scutellata*) by socially parasitic workers of *Apis mellifera capensis*. *J. Insect. Physiol.* **102**, 42–49. (doi:10.1016/j.jinsphys.2017.09.001)
78. Katzav-Gozansky T, Soroker V, Francke W, Hefetz A. 2003 Honeybee egg-laying workers mimic a queen signal. *Insectes Soc.* **50**, 20–23. (doi:10.1007/s000400300003)
79. Okosun OO, Yusuf AA, Crewe RM, Pirk CW. 2015 Effects of age and reproductive status on tergal gland secretions in queenless honey bee workers, *Apis mellifera scutellata* and *A. m. capensis*. *J. Chem. Ecol.* **41**, 896–903. (doi:10.1007/s10886-015-0630-6)
80. Princen S, Oliveira R, Ernst U, Millar JG, van Zweden J, Wenseleers T. 2019 Data from: Honeybees possess a structurally diverse and functionally redundant set of queen pheromones. Dryad Digital Repository. (https://doi.org/10.5061/dryad.s31v04d)