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# A conserved class of queen pheromones? Re-evaluating the evidence in bumblebees (*Bombus impatiens*)

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The regulation of reproductive division of labour is a key component in the evolution of social insects. Chemical signals are important mechanisms to regulate worker reproduction, either as queen-produced pheromones that coercively inhibit worker reproduction or as queen signals that honestly advertise her fecundity. A recent study suggested that a conserved class of hydrocarbons serve as queen pheromones across three independent origins of eusociality. In bumblebees (*Bombus terrestris*), pentacosane ($_C25$) was suggested to serve as a queen pheromone. Here, we repeat these studies using a different species of bumblebee (*Bombus impatiens*) with a more controlled experimental design. Instead of dequeened colonies, we used same-aged, three-worker queenless groups comprising either experienced or naive workers (with/without adult exposure to queen pheromone). We quantified three hydrocarbons ($_C23$, $_C25$ and $_C27$) on the cuticular surfaces of females and tested their effects on the two worker types. Our results indicate differences in responses of naive and experienced workers, genetic effects on worker reproduction, and general effects of hydrocarbons and duration of egg laying on ovary resorption rates. However, we found no evidence to support the theory that a conserved class of hydrocarbons serve as queen pheromones or queen signals in *Bombus impatiens*.

## 1. Background

Reproductive division of labour is a hallmark of sociality in insects, but the means by which it is regulated can vary across species. In primitively eusocial insect species with small colonies, behavioural mechanisms such as aggression are often used by the dominant female to inhibit the reproduction of the subordinate workers. In highly eusocial insect species with large colonies, chemical signals are often used. These signals allow an efficient reproductive division of labour across a large social group, reduce the use of costly behaviours that hamper the group's productivity, and provide a more efficient and precise way to regulate multiple complex behaviours [1–3].

Chemicals that inhibit worker reproduction have been identified in many species, and have been defined either as 'queen pheromones' in the cases of *Apis mellifera* [4], *Solenopsis invicta* [5] and *Nasutitermes takasagoensis* [6], or as 'queen signals' in the case of several other species [2,7–9]. In the current literature, the terms 'queen pheromones' and 'queen signals' have been used to describe two distinct evolutionary scenarios that can lead to the existence of such chemicals [3,10]. While 'queen pheromone' refers to a chemical produced by the dominant female that inhibits ovarian activation in the subordinates, presumably against their interests, 'queen signal' (also termed as 'honest signal' or 'fertility signal') refers to a chemical that varies with and advertises the fecundity of the dominant, fertile female, allowing the subordinates to gain greater fitness by remaining sterile and acting as helpers.

Distinguishing between queen pheromones and queen signals can be challenging, however, because both evolutionary scenarios lead to similar outcomes, namely the reduction of reproduction in subordinates [1–3,10]. Both scenarios

also require the chemicals to be specific and robustly detected by the subordinates. In the case of queen signals, the chemical signatures must allow for an accurate assessment of dominance [11], whereas in the case of a queen pheromone, the chemical signatures are predicted to be elaborated owing to an arms race between the dominant and subordinates [2,12].

Despite the difficulty in differentiating between the evolutionary scenarios, there are predicted differences in the patterns of production of queen pheromones and queen signals. Queen pheromones are predicted to be queen-specific and their inhibitory effects should not be correlated with the queen's egg-laying ability or mating status or be produced by the subordinates, even if they become reproductively active [10]. Alternatively, queen signals are predicted to correlate with fecundity [10,11]. As such, they can be produced by both queens and subordinates, as long as they accurately reflect their reproductive physiology. Despite these different predictions, it remains challenging to distinguish between possible queen pheromones and queen signals, because the chemical communication systems of social insects have been found to be intricate and complex. Honeybee queens, for example, produced a complex blend of chemicals from multiple glands that can modulate worker behaviour and physiology, where some of the chemicals are present at significantly higher levels in queens than workers (and some of these can also be produced by reproductive workers), other chemicals change quantity once the queen is mated (even if she is inseminated with only saline), others chemicals change quantity with mating quality and number, and finally, one chemical (homovanillyl alcohol) appears to be a dopamine mimic and thus represent a true 'coercive' pheromone [13–20].

Chemical signals are often produced by exocrine glands and secreted onto the cuticular surface, where they serve as an identification tag of the individual. Many chemical signals consist of mixtures of long chain hydrocarbons that are highly abundant and constitute the majority of any glandular secretion [21]. Hydrocarbon profiles also vary with individual species, sex, genotype, physiological and reproductive status, behavioural state and social group, and thus play an important role in insect recognition systems [21]. Thus, hydrocarbon profiles are quite flexible and well suited to serve as queen signals and to advertise changes in physiology and fecundity, but potentially are less likely to serve as queen pheromones.

Bumblebees are a well-studied model system for evaluating the factors that regulate queen–worker interactions and reproductive division of labour in a primitively eusocial species [22]. In bumblebees, reproduction is monopolized by the queen during the first half of the social life cycle, and reproductive dominance hierarchies are rapidly established among groups of queenless (QL) workers. Previous studies indicated that contact with the queen was necessary to reduce worker ovary activation rates [23,24]. Whole body washes can reduce juvenile hormone titres in workers (juvenile hormone is a gonadotropin in bumblebees [25–27]), but the identity of the chemical signals have eluded researchers [27–30].

Two recent studies performed by Holman and co-workers [31,32] found that exposing recently dequeened *Bombus terrestris* bumblebee colonies to the hydrocarbon pentacosane ($_C$25) affected reproduction in workers. However, in the two studies, ovarian activation rates were measured using non-conventional methods—classifying ovaries to developed, undeveloped or regressed [32], or counting the number of developed oocytes [31]—instead of the more commonly used approach of measuring the size of the terminal oocytes where at least three out of eight terminal oocytes in the ovaries are measured and an average is used to reflect the reproductive status of the bee (reviewed in [22]). In Van Oystaeyen *et al.*'s study [32], $_C$25 caused increased levels of ovary regression, but not decreased ovary activation rates. Regressed ovaries, also termed as 'oocyte resorption', are observed in more than 70% of the laying workers and in almost all laying queens [33]. This parameter was not examined by Van Oystaeyen *et al.* or any other study to determine if it accurately reflects the actual reproductive output of an individual worker bee and was never shown to be the result of reproductive inhibition. In Holman's study [31], exposure to $_C$25 reduced the number of 'developed oocytes', but this parameter was not well defined (e.g. what constitutes a developed versus undeveloped oocyte), nor was this shown to correlate with reproductive output (number of eggs laid or oocyte size). Furthermore, both studies did not evaluate the effects of other hydrocarbons, and the assays were performed using full colonies where many factors cannot be properly controlled, such as worker demography and density. In particular, workers in dequeened colonies would have been previously exposed to a $_C$25-producing queen and workers, making it impossible to determine if their response to the chemical was learned (as in the case of 'signature mixtures' used for nest-mate recognition) or innate (as in the case of a true pheromone [34]).

Based on these studies, it was postulated that $_C$25 is part of a conserved class of hydrocarbons used as queen pheromones across hymenopteran species [32,35]. However, as noted above, hydrocarbons seem to be unlikely candidates for 'queen pheromones' because they are so ubiquitous and variable [21]. Indeed, in *B. terrestris* bumblebees, $_C$25 is found in high quantities on the cuticular surface and in most exocrine glands of queen and workers, both fertile and non-reproductive [29,30].

Given the methodological concerns and the ubiquity of $_C$25 in bumblebees, we sought to examine, using the common North American bumblebee species *B. impatiens*, (i) whether $_C$25 levels are associated specifically with caste or fertility in *B. impatiens* females, and (ii) whether exposure to $_C$25 reduces *B. impatiens* worker ovarian activation, as expected if $_C$25 represents a conserved class of queen pheromones or queen signals. Most aspects of reproduction in *B. impatiens* are very similar to *B. terrestris*: workers in either full-sized colonies or small groups are reproductively inhibited by the presence of the queen and/or dominant workers, and workers in small QL groups form a dominance hierarchy and fully activate their ovaries and lay eggs within 7–9 days [22,36]. Unlike in the original studies, we used a controlled system of same-age, three-worker groups, of either naive workers (sampled as one-day-old callows to ensure they were not previously exposed to queen-produced odours as adults) or experienced workers (taken from queenright (QR), young colonies). We chronically exposed workers to two doses of $_C$25, corresponding to the doses used in the previous studies, and examined the effect of $_C$25 on worker egg laying latency and quantities, ovarian activation (as measured by the terminal oocyte size), and the proportion of workers exhibiting oocyte resorption. We compared this treatment with the effects of the solvent alone (hexane), and two other hydrocarbons, tricosane ($_C$23) and heptacosane ($_C$27), which we also quantified in *B. impatiens* cuticular surface. $_C$23, $_C$25, $_C$27 and $_C$29 are the four most abundant straight hydrocarbons on *B. impatiens*'s cuticle surface (E.A., M.O. & C.M.G. 2015, unpublished data). We

**Table 1.** Mean quantities of cuticular hydrocarbons (μg per individuals) in *Bombus impatiens* queens and workers. Groups were chosen according to caste (queen/workers), ovarian activation (non-reproductive/fertile) and social condition (QR/QL). Different letters following the means denote significant statistical differences (one-way ANOVA, $p < 0.001$) among different groups for each of the hydrocarbons. Data are presented as means ± s.e.

| group (*n*) | $_C$23 | $_C$25 | $_C$27 |
|---|---|---|---|
| fertile queens (15) | 13.7 ± 1.5$^a$ | 15.1 ± 2.1$^a$ | 4.38 ± 0.85$^a$ |
| virgin non-reproductive queens (10) | 6.92 ± 0.58$^b$ | 3.76 ± 0.25$^b$ | 1.73 ± 0.17$^b$ |
| QR non-reproductive workers (13) | 1.38 ± 0.12$^c$ | 1.81 ± 0.09$^b$ | 0.74 ± 0.06$^b$ |
| QL non-reproductive workers (14) | 1.31 ± 0.17$^c$ | 1.58 ± 0.15$^b$ | 0.56 ± 0.04$^b$ |
| QL fertile workers (13) | 3.46 ± 0.77$^c$ | 3.62 ± 0.64$^b$ | 0.66 ± 0.11$^b$ |

chose $_C$23 and $_C$27 as controls because they are of the closest length to $_C$25.

We hypothesized that: (i) if $_C$25 serves a queen pheromone, its levels should be queen-specific and workers, including fertile workers, should not produce it; (ii) if $_C$25 serves as queen signal its levels should vary with fertility in both queens and workers; (iii) if $_C$25 serves as a queen pheromone or a queen signal, the isolated compound should reduce ovarian activation in workers, and its effect should be distinct from the effects of other hydrocarbons of a similar length; and (iv) if $_C$25 is a learned signal, naive workers are less likely to respond to it than experienced workers.

## 2. Methods

### (a) Determining hydrocarbon quantities in *Bombus impatiens*

*Bombus impatiens* females were sampled at Pennsylvania State University: 15 fertile queens were sampled from 15 different colonies (because each colony contain only one active queen), and the rest of the females were sampled from three of these colonies. Females were shipped to Tel Aviv University, Israel for chemical analysis. Five different groups were examined. *Fertile queens* ($n = 15$): active, egg-laying queens with activated ovaries (mean oocyte size > 2 mm, corresponding to the size of 'ready to lay' eggs) of full-sized colony. *Virgin queens* ($n = 10$): approximately 7-day-old unmated queens with inactivated ovaries (mean oocyte size < 0.5 mm) from full-sized, QR colonies. *Non-reproductive QR workers* ($n = 13$): 4-day-old workers with inactivated ovaries (mean oocyte size < 0.3 mm) from full-sized, QR colonies. *Non-reproductive QL workers* ($n = 14$): 4-day-old workers with inactivated ovaries (mean oocyte size < 0.5 mm) from full-sized, dequeened colonies. *Fertile QL workers* ($n = 13$): 10-day-old workers, egg layers with activated ovaries (mean oocyte size > 2 mm) that were reared in small QL groups (3–5 workers per group). The legs of each individual were separated and extracted in 500 μl pentane containing 6 μg of eicosane ($_C$20) as internal standard. Chemical analyses were performed by gas chromatography using DB-1 fused silica capillary column (30 m × 0.25 mm ID) under a temperature programme from 60°C to 300°C at 10°C min$^{-1}$. Compound identity was ascertained by gas chromatography/mass spectrometry (GC/MS) by comparing retention times and mass signatures with synthetic compound standards. Compound quantification was achieved by GC peak integration compared with the internal standard under the same chromatographic conditions using Galaxie chromatography software.

### (b) Rearing and treatment conditions

Colonies of *B. impatiens* ($n = 7$; Koppert Biological Systems, Howell, MI, USA) were obtained two weeks after the first worker had emerged. Thus, these colonies were still young and in the worker- (not gyne-) producing phase of the colony cycle, where adult workers have inactive ovaries [30,36]. Colonies were maintained in the laboratory in nest-boxes at a constant temperature of 28–30°C and 40–50% relative humidity, and supplied ad libitum with a sugar solution and fresh pollen collected from honeybee colonies. Three-worker groups were established using either newly emerged callow workers (less than 24 h old; 'naive groups'), or random workers of unknown age ('experienced groups') that were collected from young colonies. Groups of three workers are a well-established model to test various parameters related to reproduction in bumblebee workers. Once workers are separated from the colony they will rapidly establish a dominance hierarchy and lay eggs within 7–9 days [22,37]. Callow workers can be easily distinguished from other workers by their lack of pigmentation during days 1–2 after emergence. Thus, experienced workers were at least 3 days old at the time of sampling. Callow workers cannot be distinguished among nest-mates and are readily accepted by other colonies [38]; they do not show or attract aggression and will accept any queen as well as take care of any type of brood. Thus, there is little evidence that they have learned to respond to particular chemicals associated with the queen. Older workers however can attract and/or show aggression towards unrelated workers, queens or brood. Therefore, naive groups were established using callows collected from different source colonies, and experienced groups were composed of full sisters originating from the same colony.

Naive or experienced groups were randomly assigned to one of seven treatments as follows: (i) 5 μl day$^{-1}$ of hexane; (ii) 20 ng day$^{-1}$ of tricosane; (iii) 2000 ng day$^{-1}$ of tricosane; (iv) 20 ng day$^{-1}$ of pentacosane; (v) 2000 ng day$^{-1}$ of pentacosane; (vi) 20 ng day$^{-1}$ of heptacosane; and (vii) 2000 ng day$^{-1}$ of heptacosane. All hydrocarbons were obtained from Sigma (>98% GC; tricosane: 263850; pentacosane: 286931; heptacosane: 51560), dissolved in hexane and were applied in a volume of 5 μl day$^{-1}$ onto a cotton ball that was placed in the worker cage. The high dose was chosen based on the Holman study [31] where each colony (containing approx. 300 workers) received 2000 ng day$^{-1}$. The low dose was chosen based on the proportional dose per worker in three-worker group (1 : 100 compared with the Holman study). The dose that was used in the Van Oystaeyen *et al.* study [32] (467 μg day$^{-1}$ for a young colony containing few dozen of workers, equivalent to two queens, according to the authors) is an extremely high dose compared with the known quantities of $_C$25 that a queen produces in *B. terrestris* (for example, in the Dufour's gland, 4000 ng day$^{-1}$ are produced [30]; see also table 1 below for hydrocarbon quantities in *B. impatiens*). All worker groups were kept for 10 days in small plastic cages, each lined with a filter paper and provided with a cotton ball and ad libitum sugar and pollen. Workers were collected on dry ice on day 10. Thus, at the point of collection, workers in the naive groups were 10 days old, whereas workers in

the experienced groups were 13 days old or older. For each of the groups, we collected the following data: colony source of the workers, mortality, worker body size, ovarian activation and signs of resorption in ovaries, latency to egg laying and the cumulative number of eggs found in the cage by the end of the experiment.

### (c) Mortality

If one or more bees in a group died during the experiment, the entire group was excluded from the analysis, to ensure the dose applied per worker remains constant and data on reproduction is not affected by the number of workers [39]. Overall, we established 204 groups of workers. Among these, 14 were excluded, resulting in 190 full groups (570 workers) that were used in the final analysis. Overall, mortality was very low (approx. 2%) and was not associated with any of the treatments or worker type.

### (d) Worker body size

Worker body size was examined individually by measuring the width of the head capsule between the two compound eyes under a stereo microscope [39].

### (e) Ovarian activation and signs of resorption

After bees were sampled, each bee was placed in a separate tube and received an individual number. Thus, analysis of ovarian activation was performed blindly. Individual bees were dissected under a stereo microscope in double-distilled water. The length of the terminal oocyte in the three largest ovarioles (at least one ovariole per ovary; workers possess four ovarioles per ovary) was measured with a scaled ocular. Mean terminal oocyte length for each bee was used as an index of ovarian activation. Resorption was defined by deformations in the terminal oocytes accompanied by a change in its colour. Resorbed oocytes often look thicker and shorter and exhibit a yellow-grey colour instead of the typical shiny white of mature oocytes [33]. Cases where the signs for resorption were not clear (3% of the cases) were excluded from the final analysis.

### (f) Egg laying

All cages were scanned for the presence of newly laid eggs every morning during the 10 days of the experiment in order to document the first day where egg laying was observed in each cage. The cumulative number of both eggs and larvae were counted on the day of collection (day 10). While egg oophagy generally exists in bumblebees [40], it is often performed in QR colonies and rarely occurs in small QL groups [22]. We did not see evidence for oophagy (such as open egg cells, etc.) that could affect the results.

### (g) Statistics

The differences in quantities of hydrocarbons in the different queen and worker groups were examined using a one-way ANOVA followed by Tukey-type *post hoc* test. The effect of treatment and worker type on terminal oocyte size, latency to egg laying, cumulative number of eggs and body size was examined using two-way ANOVA with treatment (identity of hydrocarbon and dose) and worker type (naive or experienced) as factors. Power analysis for the sufficient sample sizes for the terminal oocyte size and number of eggs was performed using the software G*POWER. The proportion of resorption was compared using Pearson's chi-squared test of observed versus expected. Colony identity was examined in experienced worker groups using a two-way ANOVA with treatment (identity of hydrocarbon and dose) and source colonies as factors. Correlations were tested using Pearson correlation. Statistical analyses were performed using STATISTICA for Windows, v. 12.0. Statistical significance was accepted at $\alpha = 0.05$. Data are presented as means $\pm$ standard errors.

## 3. Results

### (a) Hydrocarbon quantities in *Bombus impatiens* queens and workers

Chemical analysis of the cuticular surface in queens and workers (table 1) revealed that $_C$23, $_C$25 and $_C$27 are all highly abundant in both queens and workers, regardless of their social condition (QR or QL) or their level of ovarian activation (non-reproductive or fertile). The quantities of all three hydrocarbons were significantly higher in fertile queens compared with the virgin queens and the three groups of workers (QR non-reproductive, QL non-reproductive, QL fertile). In the case of $_C$23, there were also significant differences between virgin queens and the three groups of workers. Although fertile workers produced slightly higher quantities of $_C$23 and $_C$25, there were no significant differences in the quantities among the different worker groups, even when there were clear differences in their ovarian activation. The results of the statistics are provided in electronic supplementary material, table S1. Overall, the chemical results were very similar to the findings reported for *B. terrestris* in the Van Oystaeyen *et al.* study [32].

### (b) Ovarian activation

Most of the workers in the study had fully developed ovaries, regardless of the treatment, with 62% of the workers ($n = 569$) having average terminal oocyte larger than 2 mm (indicating 'ready-to-lay' eggs) and at least one worker having terminal oocyte larger than 2 mm per group in 94% of the groups ($n = 190$).

Ovarian activation was measured in two different groups of workers (10 day old, naive versus >13 day old, experienced) and in seven different treatments (low and high dose of $_C$23, $_C$25, $_C$27 versus hexane; figure 1). Worker type but not the treatment had a significant effect on the average terminal oocyte size of workers, and no interaction was found between worker type and treatment (statistics are provided in electronic supplementary material, table S2). Surprisingly, in all cases, ovarian activation by naive workers was significantly higher compared with the older experienced workers.

### (c) Egg laying

Eggs hatch to larvae approximately 5 days after they are laid [36,41]. Thus, the latency to egg laying affected not only the cumulative number of eggs found in the cage by the end of the experiment (the earlier eggs were laid, the more eggs found on day 10; Pearson correlation: $n = 185$, $r = -0.4$, $p < 0.001$), but had also affected the type of brood (eggs or larvae) that was found in each cage.

There were significant differences in the latency to first egg laying in the different worker types, but not in the different treatments, with the experienced workers laying eggs significantly earlier compared with the naive workers (statistics are provided in electronic supplementary material, table S3).

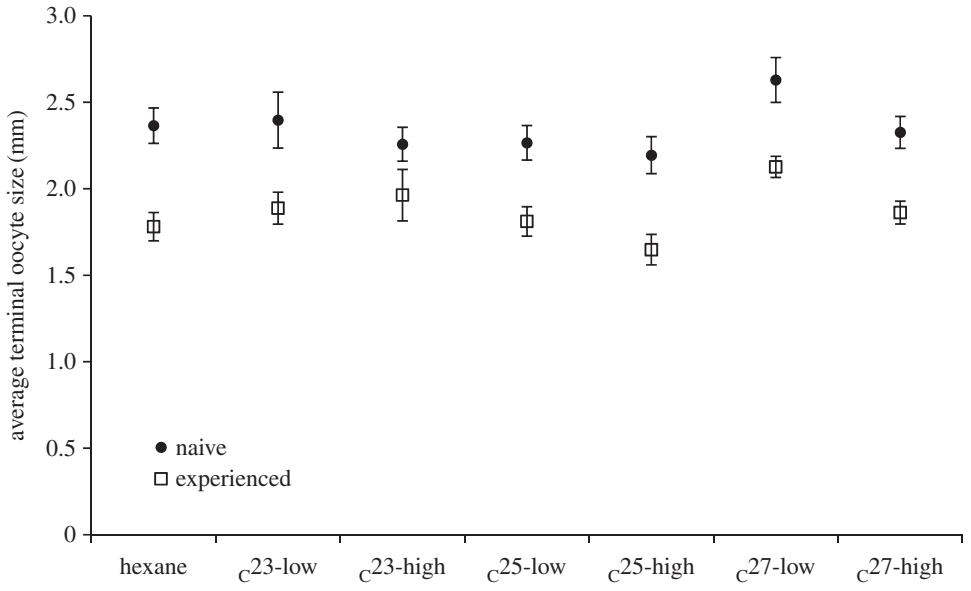


**Figure 1.** Average terminal oocyte size (mm) in two types of workers assigned to seven different treatments. Workers ($n = 569$, 18–69 workers per treatment group) were sampled from source colonies ($n = 7$) as either naive (when they were less than 24 h old) or as experienced (at the age of 3 days or older from young colonies where queen reduces worker reproduction). Workers were then kept in groups of three for 10 days during which they were exposed daily to either hexane (control solvent) or low dose (20 ng day$^{-1}$) or high dose (2000 ng day$^{-1}$) of tricosane ($_C$23), pentacosane ($_C$25) or heptacosane ($_C$27). Chemicals were applied daily onto a cotton ball in a volume of 5 µl. Data are presented as means ± s.e.

Although eggs were laid in most of the groups, regardless of the treatment, there was substantial variation in the number of eggs laid in the different groups and treatments (figure 2). While there were no significant differences in the number of eggs in the different treatments or worker type (statistics are provided in electronic supplementary material, table S4), there was a significant interaction between the two, but no significant difference for hexane versus all treatments.

Because we report negative results that contradict previous studies, we also ensured that our sample size was sufficient for the hypotheses we tested by performing *post hoc* power analysis for the effect of treatment on the average terminal oocyte size and the number of eggs. A sample size of 570 workers and 190 groups had a power of 99% and 96% for effect size of 0.2 and 0.5, respectively. Thus, if $_C$25 was a true pheromone and could at least reduce ovary size in 20% or eggs laid in 50%, the current sample size would yield a significant result in 96–99% of the time. Tables describing the relation between sample size, effect size and power for ovarian activation and egg laying are provided in the electronic supplementary material, table S5a,b.

## (d) Resorption

Resorption was defined by a change in the shape or the colour of the terminal oocytes (see Methods). Because it is the major effect shown to occur in response to $_C$25 exposure in *B. terrestris* workers [32], we further examined its occurrence and interactions with other factors such as worker type, treatment and the presence of brood. The percentages of resorption in hexane-treated groups were 18% and 25% for naive and experienced workers, respectively (table 2). We used these percentages to calculate the expected versus observed percentages of resorption in workers in each one of the other treatments. In all treatments, the resorption was higher than expected relative to the solvent controls, and often was two to three times higher than in the presence of the solvent alone (for naive workers: $\chi^2 = 62.74$, d.f. = 5 $p < 0.001$; for experienced workers: $\chi^2 = 42.8$, d.f. = 5, $p < 0.001$; table 2).

Resorption was also higher in groups of experienced versus naive workers, and in response to different brood types. Eggs were laid earlier in groups of experienced versus naive workers (day 6 ± 0.14 versus day 8 ± 0.2, on average, based on data collected in 175 groups), thus hatched to larvae in many of the experienced groups but did not hatch in any of the naive groups. We found a significantly highly negative correlation ($r = -0.86$, $p = 0.002$) between the day where eggs were laid (2–10) and the percentage of resorption rate (figure 3).

## (e) Body size

Worker body size was found to be positively associated with ovarian activation in a previous study [31,42]. Because we randomly grouped workers, we verified that their body size was not a cofounding factor that affected ovarian activation in the different treatments. Indeed, body size did not differ in the different treatments (statistics are provided in electronic supplementary material, table S6). However, the naive workers were significantly larger than the experienced workers (table 2).

Body size was also positively correlated with ovarian activation in workers with a linear regression providing the best fit to describe the relationship between the two parameters. However, although the correlation was highly significant ($n = 567$, $p < 0.001$), it was not very strong ($r = 0.23$). This was due to what appears to be a threshold in worker body size: workers with a body size larger than 4 mm (= the length between the two compound eyes; sizes ranged between 2.72 and 4.42 mm) showed higher ovarian activation and substantially less variance compared with workers with body size smaller than 4 mm (>4: 2.38 ± 0.5 mm, $n = 60$; < 4:

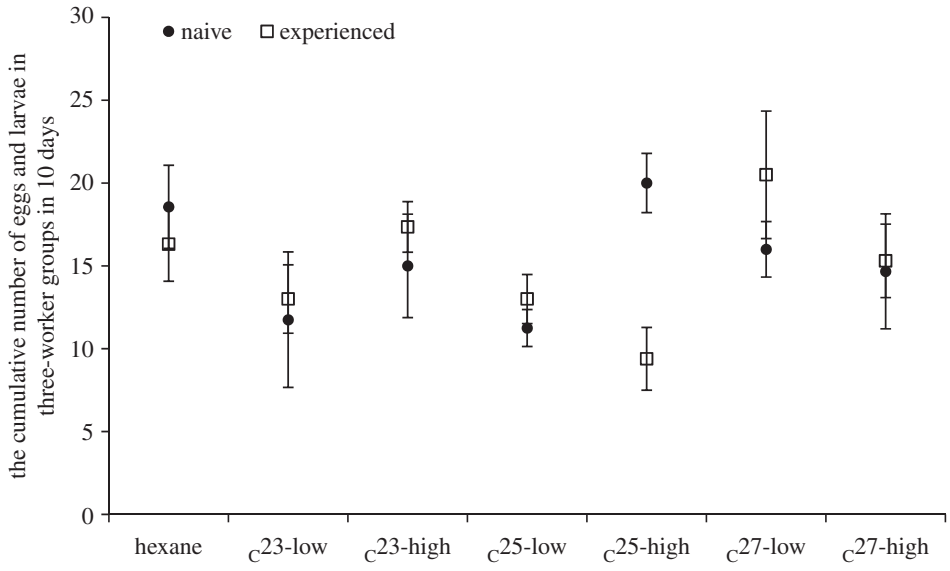


**Figure 2.** Cumulative number of eggs and larvae laid by three-worker groups in 10 days. Egg laying was examined in two types of workers assigned to seven different treatments ($n = 190$ groups, 6–23 groups per treatment). Workers were sampled from source colonies ($n = 7$) as either naive (when they were less than 24 h old) or as experienced (at the age of 3 days or older from young colonies where queen reduces worker reproduction). Workers were then kept in groups of three for 10 days during which they were exposed daily to either hexane (control solvent) or low dose (20 ng day$^{-1}$) or high dose (2000 ng day$^{-1}$) of tricosane ($_C$23), pentacosane ($_C$25) or heptacosane ($_C$27). Chemicals were applied daily onto a cotton ball in a volume of 5 µl. Data are presented as means ± s.e.

**Table 2.** Latency to egg laying, body size and percentage of resorption in two types of workers (naive and experienced) assigned to seven different treatments. Data are presented as means ± s.e. Sample size for each group is the same as in figures 1 and 2.

| | latency to egg laying (days) | | body size (mm) | | oocyte resorption (%) | |
|---|---|---|---|---|---|---|
| | naive | experienced | naive | experienced | naive | experienced |
| hexane | 7.93 ± 0.4 | 5.4 ± 0.4 | 3.65 ± 0.05 | 3.61 ± 0.03 | 17.8 | 25 |
| $_C$23-low | 8.75 ± 0.7 | 5.3 ± 0.6 | 3.49 ± 0.09 | 3.51 ± 0.04 | 58.3 | 20 |
| $_C$23-high | 8.5 ± 0.2 | 5.8 ± 0.4 | 3.54 ± 0.08 | 3.71 ± 0.05 | 55.6 | 50 |
| $_C$25-low | 8.33 ± 0.2 | 6 ± 0.4 | 3.71 ± 0.04 | 3.52 ± 0.04 | 32.4 | 45 |
| $_C$25-high | 7.66 ± 0.3 | 7.7 ± 0.6 | 3.66 ± 0.06 | 3.53 ± 0.03 | 39.4 | 34.8 |
| $_C$27-low | 7.5 ± 0.7 | 4.2 ± 0.5 | 3.58 ± 0.07 | 3.44 ± 0.05 | 44.4 | 53.3 |
| $_C$27-high | 8 ± 0 | 6.7 ± 0.6 | 3.77 ± 0.08 | 3.58 ± 0.04 | 55.6 | 39.3 |
| statistics | n.s. between treatments[a] $p < 0.001$ for worker type no interaction | | n.s. between treatments[a] $p = 0.02$ for worker type no interaction | | chi-square $p < 0.001$ for observed versus predicted (based on control levels) in both worker types | |

[a]Two-way ANOVA; for further details please refer to the text and to the electronic supplementary material.

1.95 ± 0.77 mm, $n = 507$). However, only about 10% of the workers reached a body size larger than 4 mm, suggesting that body size affects ovarian activation only in the upper extreme body size of workers and is not playing an important role for the majority of the workers.

## (f) Colony identity

Seven different colonies were used to provide workers for this study. However, only three of the colonies produced enough cages of workers to robustly examine the effects of the colony identity on the examined parameters. We therefore used cages produced from these three colonies (colonies E, F and G; $n = 207$ workers) to examine whether a bias in colony identity exists and whether it affects ovarian activation, egg laying (cumulative number and latency to lay eggs) or resorption in workers. Additionally, because groups of naive workers were always a mix of different source colonies (see Methods), we examined only a possible bias in groups of experienced workers. When we used this set of data, we found again no significant differences in ovarian activation, latency to egg laying and cumulative number of eggs as function of the treatment

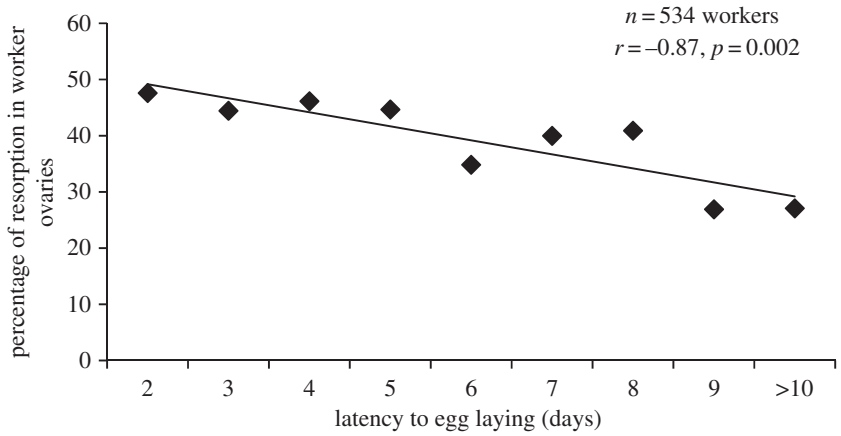


**Figure 3.** The relation between latency to egg laying and the percentages of resorption in worker ovaries, regardless of treatment or worker type. Workers ($n = 534$) were sampled from seven source colonies as either naive (when they were less than 24 h old) or as experienced (at the age of 3 days or older from young colonies where queen reduces worker reproduction) and were subjected to one of the seven treatments. The proportion of resorption was negatively correlated with the timing of first egg laying in the cages.

**Table 3.** Colony identity impact on reproduction. Effect of colony source on the average terminal oocyte, latency to egg laying and cumulative number of eggs in experienced workers assigned to seven different treatments in three out of the seven source colonies that were used in this study. Sample size for workers/groups for each colony source are: E: 26/78; F: 13/39; G: 30/90.

| | colony source | | | statistics (two-way ANOVA) | | |
|---|---|---|---|---|---|---|
| | E | F | G | treatment effect | colony source effect | interaction |
| average terminal oocyte size (mm) | 2.1 ± 0.08 | 1.86 ± 0.1 | 1.66 ± 0.1 | $p = 0.38$ | $p = 0.001$ for G versus E and F | $p = 0.78$ |
| latency to egg laying (days) | 4.65 ± 0.3 | 6.53 ± 0.6 | 5.42 ± 0.4 | $p = 0.04$[a] | $p = 0.02$ for E versus F | $p = 0.36$ |
| cumulative number of eggs | 18.9 ± 0.1 | 13.2 ± 2.7 | 9.6 ± 1.5 | $p = 0.07$ | $p < 0.001$ for E versus G | $p = 0.44$ |
| percentage of resorption | 42.3 | 30.7 | 37.7 | | | |

[a]*Post hoc* test revealed $p > 0.05$ for hexane versus all other treatments.

(statistics are provided in table 3 and electronic supplementary material, table S7). However, in all cases, we found a significant effect by the colony source, suggesting all these parameters are affected by colony identity (statistics are provided in table 3 and electronic supplementary material, table S7). No interaction was found between treatment and colony source in any of the cases (statistics are provided in table 3 and electronic supplementary material, table S7). The percentages of resorption too were substantially different in the different colonies (42.3%, 30.7%. 37.7% in workers from source colonies E, F and G, respectively).

## 4. Discussion

In terms of relative abundance in *B. impatiens* queens and workers, none of the three tested chemicals ($_C$23, $_C$25 and $_C$27) fully match the classical definition of a queen pheromone (produced at higher quantities in queens versus workers, regardless of the queen's reproductive status) or a queen signal (produced at higher quantities in fertile queens/workers versus sterile queens/workers). All of the chemicals were produced at significantly higher levels in mated, laying queens versus virgin queens, and there was a non-significant trend for $_C$23 and $_C$25 to be produced at higher levels in laying workers versus sterile workers. These results are qualitatively similar to the data obtained in *B. terrestris* in the Van Oystaeyen *et al.* study [32]. Thus, based on relative abundance, none of the three hydrocarbons meet the definition of a queen pheromone and only two (assuming they can also reduce ovarian activation in workers) partially meet the definition of a queen signal.

A queen pheromone or queen signal should reduce worker reproduction, and our data did not show any evidence that any of the three tested hydrocarbons reduced *B. impatiens* worker reproduction. The three hydrocarbons (relative to the solvent control) had no effect on worker reproduction in either the naive or experienced *B. impatiens* workers, based on worker oocyte size, latency to lay eggs and the cumulative number of eggs. This is in contrast to the Holman study [31] which did find a reduction in the number of developed oocytes, though it was not clearly explained how oocyte size was assessed, but consistent with the Van Oystaeyen *et al.* study [32], which found no effect on ovarian activation.

The only parameter that was affected by the treatment was the proportion of oocyte resorption, which is consistent with the Van Oystaeyen *et al.* study [32]. However, the rate of resorption was not related to oocyte size or the amount of eggs laid during the time scale of the experiment

(10 days). Resorption rates were, however, negatively related to the time at which egg laying was initiated in a particular cage, and thus could perhaps simply reflect the duration of egg production in the workers or the presence of developing brood. Indeed, previous studies found that resorption is seen in most laying queens and workers, and could simply be a normal physiological response to extended period of egg laying [33]. Thus, it is unclear how resorption could serve as a reliable parameter to measure *active* inhibition of reproduction by a chemical signal. Furthermore, because the effects on resorption rates were found with all three hydrocarbons, the effects are either non-specific or all three hydrocarbons are serving as subtle regulators of worker reproductive physiology. Testing a hydrocarbon which is not found on the cuticles of queens would distinguish between these possibilities. Overall, based on both the chemistry and the bioassay, our results suggest that $_C$25 (as well as $_C$23 and $_C$27) does not act as a queen pheromone or queen signal in *B. impatiens* bumblebees, and call into question the existence of a conserved class of queen pheromones across hymenopteran species.

Our study did reveal differences in the patterns of reproduction between naive and experienced workers. Although naive workers were younger than the experienced workers, their ovaries (on average) were significantly more activated (figure 1). However, experienced workers initiated egg laying earlier (table 2), and both naive and experienced groups laid the same number of eggs (except of a single difference that is discussed below; figure 2). The naive workers exhibited significantly larger body sizes (table 2), and because body size is positively associated with ovary activation rates [31,42], this may explain the differences between the two groups. Interestingly, despite the earlier onset of egg laying, the experienced workers did not lay more eggs overall, and thus it is possible their egg-laying capacity was reduced by age, such that experienced workers laid fewer eggs per day. A shorter latency to lay eggs in the experienced workers (possibly caused by their advanced age) suggests that in colonies, experienced workers are better prepared for an opportunity to reproduce compared with non-reproductive callow that just emerged.

There was, however, a significant difference in egg-laying behaviour by naive versus experienced workers that were treated with the high dose of $_C$25. Naive workers treated with the high dose of $_C$25 laid significantly more eggs than the experienced workers treated with the high dose of $_C$25 (figure 2). Egg laying in the two groups did not differ from egg laying in the solvent treatment, so this finding does not represent an inhibitory effect by $_C$25, but a more subtle difference in the threshold response to it between the two group types, presumably owing to differences in the workers' experience. Experienced workers had a greater opportunity to learn the odours of their source colony, and potentially associate these odours with the social phase where they normally refrain from egg laying [36]. $_C$25 is present at high levels in the laying queen and also produced by all workers (table 1); thus, it is possible that the high dose of $_C$25 mimicked the odour of the source colony, serving as a learned signal to reduce worker reproduction. Notably, there was no difference in responses of naive and experienced workers to the high dose of $_C$23 and $_C$27. However, $_C$25 is far more abundant than $_C$23 and $_C$27 in *B. terrestris* [29,30,43], and in *B. impatiens* too the quantities of $_C$25 were 3.44 times higher compared with $_C$27 and only slightly higher (1.1-fold) compared with $_C$23 (table 1).

It is intriguing that exposure to all three hydrocarbons, compared with the solvent, increased rates of oocyte resorption in fertile workers. As discussed above, oocyte resorption is common in egg-laying queens and workers, and may simply reflect the length of time an individual has been laying or the amount of brood present [33]; indeed, our results show that resorption rates are negatively correlated with the time to initiate egg laying. However, the three hydrocarbons did not affect time to initiate egg laying, and thus this does not seem to be a direct effect of the hydrocarbons on ovary activation and egg laying. Interestingly, all three hydrocarbons are highly abundant on the egg surface in *B. terrestris* [44], and thus could serve a signal of the presence of developing brood, and potentially serve to induce workers to reduce egg-laying rates. Alternatively, the hydrocarbons may have produced some low-level toxicity that triggers oocyte resorption in workers. In either scenario, oocyte resorption should lead to a reduction in the total number of eggs laid by workers, which was not observed in our studies, though perhaps this would have been apparent at a longer time scale. Regardless, even if oocyte resorption serves as a mechanism for an individual worker to reduce or adjust her egg-laying rate in response to other cues, it is not likely to be an indicator of active reproductive inhibition, as suggested by Van Oystaeyen *et al.* [32].

Finally, our data demonstrated that worker reproduction is affected by colony identity. Workers from different colonies varied significantly in ovary activation rates, latency to lay eggs, and the cumulative number of eggs laid (table 3). While genotypic differences in worker reproduction rates have been observed in honeybees [45,46], these have not been previous observed in bumblebees.

Overall, these results indicate that $_C$25 and the other two hydrocarbons do not modulate worker reproduction in *B. impatiens*, and thus do not serve as a queen pheromone or queen signal in this species. Thus, the previous results in *B. terrestris* could not be generalized and should be re-evaluated using additional hydrocarbon controls and methods to evaluate worker reproduction. Specifically, in *B. terrestris*, we recommend consideration of the ample evidence suggesting reproduction is monopolized through a combination of chemical and behavioural means and depends upon worker density rather than depend solely on pheromones [27,39]. The gradual increase in worker population during the annual colony cycle of bumblebee species may slowly reduce the effectiveness of the queen aggressiveness and odour (as determined by her overall glandular secretion or by pheromone components that are yet to be identified), allowing the workers to escape her dominance and reproduce according to their own self interests.

Authors' contributions. E.A. designed and performed research, analysed data and wrote the paper. M.O. quantified hydrocarbons on the cuticular surfaces of *B. impatiens* females. C.M.G. designed research and wrote the paper along with E.A.

Competing interests. We have no competing interests.

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