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RESEARCH ARTICLE

Turning workers into false queens: the role of exogenous pheromones in regulating reproduction in worker honey bees

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ABSTRACT

One of the responses that honey bee workers can make in the event of queen loss is to develop into false queens. False queens are workers that exhibit both behavioural and physiological traits similar to those of a true queen. However, the presence of more than one false queen in a colony distorts the established hierarchies. As transformation into a false queen occurs after emergence as an adult, we tested the effect of worker mobile pheromone carriers (PCs) treated with exogenously supplied pheromones on their nestmates. The PCs carried either synthetic mandibular gland pheromones or pheromones extracted from *Apis mellifera capensis* parasitic workers. Only the PCs attracted retinues of workers, increased pheromone production and activated their ovaries, becoming false queens. Pheromones from *A. m. capensis* workers were more effective than extracts of commercially available synthetic queen pheromones in eliciting these effects. Using this simple mobile pheromone delivery system, we have shown that carrying amounts of exogenous pheromone can induce pheromone production in the carrier, resulting in the production of false queens within experimental groups. Possible implications of using this technique to modify and regulate worker reproduction in colonies are discussed.

KEY WORDS: Queen mandibular gland pheromones, *Apis mellifera capensis*, Pseudo queens, Pheromone carriers, Pheromone delivery system

INTRODUCTION

Reproductive division of labour is a key characteristic of eusocial insect societies. One such example is found in honey bees, where differentiation between the queen and worker castes is key to organisation in their colonies. In circumstances where this relationship is altered as a result of queen loss, workers reportedly exhibit behaviours aimed at restoring reproductive hegemony by reproductive individuals. These behaviours include an excited roaring by workers a few hours after dequeening, construction of emergency queen cells, appearance of laying workers and appearance of false or pseudo-queens (Sakagami, 1958). A false queen is usually a normal adult worker honey bee aged between 10 and 30 days; she does not undertake worker-related tasks and she exhibits some features of a queen including worker retinue attraction, egg-laying and, to some extent, regulation of ovarian activation and oviposition in other workers (Sakagami, 1958). She also possesses and secretes queen-like mandibular gland (MDG) pheromonal signals (Crewe and Velthuis, 1980).

The presence of false queens was first observed by Park (1949), and later Lundie (1954) made similar observations in the Cape honey bee (*Apis mellifera capensis* Eschscholtz 1822), a subspecies endemic to Southern Africa that is capable of producing female offspring from unfertilised workers' eggs through thelytokous parthenogenesis (Hepburn and Crewe, 1990, 1991; Onions, 1912; Ruttner, 1977).

Pheromones from the queen's mandibular glands are implicated in the control of both behavioural and physiological activities within the colony. These pheromones signal the presence of the queen, and inhibit queen rearing and activation of ovaries in other females (Butler, 1959; Slessor et al., 1998). The chemistry of the mandibular gland (MDG) and its behavioural active chemical components have been studied in great detail (reviewed in Pirk et al., 2011). Secretions from the MDG of queens are made up of two aromatic components [methyl *p*-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (HVA)] together with four fatty acids [9-oxo-2*(E)*-decenoic acid (9-ODA) and its precursor (*R,E*)-9-hydroxy-2-decenoic acid (9-HDA), (*S,E*)-9-hydroxy-2-decenoic acid (9-HDA), 10-hydroxy-decanoic acid (10-HDAA) and 10-hydroxy-2*(E)*-decenoic acid (10-HDA)] (Slessor et al., 1988; Winston et al., 1989). In contrast, those from the glands of non-laying workers are dominated by two fatty acids: 10-HDA and its precursor 10-HDAA. Together, the active pheromone components from the MDG of queens are collectively referred to as queen mandibular gland pheromones (QMPs).

The production and synthesis of MDG components is clearly dependent on several factors, including: caste, where queens produce larger quantities in comparison to workers; social context and position of the worker in queenless groups (where dominant individuals could arise); and the production of the queen substance 9-ODA, especially in the MDGs of workers that are not in direct synchrony with ovarian activation (Crewe and Velthuis, 1980). Secretions from MDGs of false queens are characterised by larger amounts of the queen substance 9-ODA, its precursor 9-HDA and the presence of ω-hydroxylated fatty acids, which are the main components in the MDG of workers (Crewe and Velthuis, 1980; Okosun et al., 2017). The false queens have the morphological characteristics of workers but the pheromonal characteristics of mated queens, thus making them distinguishable from laying and non-laying workers on the one hand and true queens on the other.

Given the unique place that false queens occupy in queenless colonies and the fact that they exemplify the modifiable physiology and behaviour of adult worker honey bees, we decided to explore the role of pheromones in their genesis. We conducted experiments to explore the potential of making false queens from workers of two subspecies of honey bees: *A. m. scutellata*, a subspecies in which laying workers are rarely produced, and *A. m. capensis* clones, which readily transform into laying workers and false

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Received 5 December 2017; Accepted 15 May 2018

Journal of Experimental Biology (2018) 221, jeb175505. doi:10.1242/jeb.175505

queens in their native colonies as well as in colonies of other honey bee subspecies (Härtel et al., 2006a,b; Moritz et al., 2003). Using simple laboratory manipulations, we applied exogenous pheromone extracts to adult honey bee workers, thus turning them into mobile pheromone carriers (PCs), and explored the effect of this pheromone on both the carriers and their nestmates. Using this setup, we tested whether MDG extracts from a synthetic and commercially available QMP supplement (Pseudo Queen®) and natural MDG extracts from *A. m. capensis* parasitic clones could transform adult workers into false queens. We also hypothesised that extracts from both sources would act similarly in this transformation.

MATERIALS AND METHODS

Honey bee rearing

Sealed brood of *A. m. scutellata* was collected from three hives at the University of Pretoria apiary (25°44′49″S, 28°15′40″E) whereas those of *A. m. capensis* clones were collected from three hives at a commercial apiary in the Gauteng region, South Africa. These were incubated separately at 34°C and 75–80% relative humidity using standard rearing methods (Williams et al., 2013) until workers emerged.

Experimental cages

Groups of workers (100) were placed in hoarding cages made of Perspex® (Köhler et al., 2013) and reared in an incubator under conditions similar to those described above for brood rearing, for a period of 14 days. This was to allow enough time for the bees to activate their ovaries, as honey bee workers under queenless conditions are known to start activating their ovaries when they are as young as 10 days old (Velthuis, 1970; Ruttner and Hesse, 1981; Jarosch-Perlow et al., 2018).

Bees were fed on sugar water (30% sucrose solution), pollen and water ad libitum. In total, 30 cages were made, each containing PCs, of which four PCs were lost during the experiments, leaving 26 PCs. Thus, at the end of the experiments we had 16 cages (16 PCs) for *A. m. scutellata* and 10 cages (10 PCs) for *A. m. capensis*.

Synthetic QMP pheromone carriers

Upon emergence, one worker per cage of either *A. m. scutellata* or *A. m. capensis* clones was uniquely marked using permanent markers on the abdomen, and a narrow cylindrical strip of Pseudo Queen® (Contech Enterprises Inc., Victoria, Canada) with synthetic QMP weighing ∼11 mg was glued to its thorax (Fig. S1). This served as a source of synthetic pheromone and this cohort of bees served as PCs. To serve as a control and check for the effect of marking, 15 workers were uniquely marked on either their thorax or abdomen in a similar way to the PCs, but no synthetic QMP was placed on their thorax. These individuals served as dummy PCs. Another group of 84 workers was not marked, and these served as the control group.

Synthetic QMP and clone MDG pheromone carriers

To compare whether extracts from clone MDGs elicit effects similar to those of an exogenous pheromone supplement source in queenless workers of *A. m. scutellata*, narrow strips of Pseudo Queen® (∼11 mg) and heads of clones were each extracted in 100 µl of dichloromethane (DCM) for at least 24 h. The composition (proportions) and amounts (concentrations) of MDG components of both extracts were at first determined using gas chromatography as described in below ('Pheromone analysis'). To prepare the pheromone delivery sources for these extracts, 3×3×3 mm cubes were made out of an 11 mm thick Thermogreen® LB-2 gas chromatograph solid disk septum (Sigma-Aldrich, catalogue no. 20654) (Fig. S2). These cubes were baked in an oven at 300°C for 4 h and thereafter impregnated with either the Pseudo Queen® components or cephalic extracts from clones by immersing them in a vial containing the extract. The solvent was allowed to evaporate from the vials and the cubes were then glued onto the thoraces of the PCs (see Fig. S2) similar to as described for Pseudo Queen® strips. As a control for the cubes, another set of cubes that were not impregnated with extracts was glued onto marked worker bees (dummy PCs). The experimental design was similar to those described for experiments with Pseudo Queen® strips. In brief, 42 PCs (19 carrying extracts from Pseudo Queen® and 23 carrying extracts from *A. m. capensis* clones) were made. Prior to this, the efficacy and durability of the cubes as a slow and clean pheromone delivery source was determined by incubating the impregnated septa under conditions similar to those used for rearing bees for a period of 21 days. The cubes were then re-extracted in DCM, and analysed on a gas chromatograph to confirm the presence and composition of MDG pheromones.

Pheromone analysis

For bees at the age of 14 days, workers were immobilised on ice, their heads were removed and cephalic extracts were made by placing the heads in 200 µl of DCM following the methods described in Yusuf et al. (2015). For Pseudo Queen® strips and septa, each was extracted in 100 µl of DCM for 24 h. Prior to gas chromatographic analysis, back-ups were made of each extract by taking half (∼100 µl for bee heads and 50 µl for strips and septa) and storing this in a freezer at −20°C until required. The other half was transferred into a GC vial and evaporated to dryness under a gentle stream of nitrogen. The resulting residue was re-dissolved in 10 µl of internal standard solution [containing ∼1 mg each of octanoic acid (C8) and *n*-tetradecane (C14) in 4 ml DCM] to which 10 µl of derivatisation agent bis-(trimethylsily) trifluoroacetamide (BSTFA) was added and then allowed to derivatise for at least 4 h.

Aliquots (1 µl) of the derivatised extracts were injected into a gas chromatograph (Agilent 6890N) fitted with a flame ionisation detector (FID) and an HP1-MS capillary column (25 m×0.20 mm×0.33 µm). Helium was used as the carrier gas at a flow rate of 1 ml min^{-1}, and oven temperature programmed as follows: 50°C at 1 min then increased to 100°C, ramped at 3°C min^{-1} to 220°C, and then held at 220°C for 10 min. Injector and FID temperatures were set at 250°C and 310°C, respectively. All recorded chromatograms were processed on a PC equipped with Chemstation® software version B.02.01 (Agilent Technologies, Waldbronn, Germany). Peaks for the six major components from honey bee MDG (HOB, 9-ODA, HVA, 9-HDA, 10-HDAA and 10-HDA) were identified based on comparison with retention times of synthetic standards and quantified using their relative mass ratios (RMR) in relation to those of the internal standards. All reagents and standards were of analytical grade and were purchased from Sigma-Aldrich.

Dissections and assessment of ovarian activation status

Ovaries were dissected from all PCs [26 carrying synthetic QMP + 42 carrying extracts of synthetic QMP (19) and those from *A. m. capensis* clones (23)=68], and from another 116 non-PCs randomly selected from each treatment. The level of ovarian activation was assessed and ranked following Hess (1942) and Schäfer et al. (2006), where workers with thread-like ovarioles were classified as undeveloped (UD) and those with oocytes in the early development or fully developed stages were classified as having

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developed ovaries (FD). The presence or absence of spermathecae were also recorded.

Statistical analyses

Data obtained for MDG components from synthetic QMP carriers (PC) non-carriers, extracts from Pseudo Queen® strips and septa were not normally distributed. Hence, non-parametric statistical tests were applied. Mann–Whitney *U*-test (MWU) was used to determine differences in the individual as well as total MDG components between PCs and other workers (control) using the treatment as the independent grouping for both amounts and proportions. For the PCs carrying extracts of synthetic QMP (Pseudo Queen®) or those with extracts from *A. m. capensis* clones, a *t*-test for independent groups was used to determine the differences in the amounts of MDG components as well as the total extracts. A chi-square (χ^2) or a Fisher's exact test (when frequencies are lower than 5) was performed to assess whether there were differences in ovarian activation status of PCs between *A. m. capensis* clones and *A. m. scutellata* carrying synthetic QMP and those of workers carrying clone and synthetic QMP extracts. Unless stated, all values on figures and tables are presented as means±s.e.m. All statistical tests were conducted using the software STATISTICA 12 (StatsSoft Inc., Tulsa, OK, USA), at a *P*-level of 0.05.

RESULTS

Mandibular gland pheromones from PCs versus non-carriers

In total, 26 (16 *A. m. scutellata* and 10 *A. m. capensis* clones) PCs carrying strips of synthetic QMPs were analysed. All the six major components of mandibular gland origin were detected in the cephalic extracts of the bees, with HVA in trace amounts from all PCs as well as *A. m. capensis* clones (Fig. 1). To eliminate the possibility that the queen substance components HOB, 9-ODA and 9-HDA were translocated from the synthetic QMP on the thoraces of PCs, their thoraces were washed and analysed for the presence of these components. Results showed that none of the MDG components were present in the thoracic extracts (Table S1).

In comparison, the amounts and proportions of MDG components in marked workers (non-pheromone carriers) and un-marked workers were not significantly different from each other (MWU, $P>0.05$, $n=23, 40$). This showed that marking had no effect on the biosynthetic activity of the MDGs of the bees. Thereafter, 25 marked and 25 unmarked bees were randomly selected, and the results were pooled together and analysed as non-PCs.

The individual components of MDG from cephalic extracts of *A. m. scutellata* PCs showed an increase in the proportion of 9-ODA (Fig. 1A) with a decrease in proportion of the worker component 10-HDAA (MWU, $P<0.05$, $n=16, 50$), which is an indication of a shift from worker-like secretions towards those that are queen-like, thus becoming false queens. In *A. m. capensis* clones, PCs had profiles similar to those of non-PCs, except for the significant increase in the proportion of 9-ODA and a decrease in its precursor (9-HDA) and in the worker substance 10-HDA (MWU, $P<0.05$, $n=10, 50$; Fig. 1B), making them more queen-like in comparison to non-PCs.

Total median pheromone production for PCs and non-PCs in *A. m. scutellata* was 3.03±1.0 and 1.95±0.95 µg, respectively, with all components except the worker component 10-HDA being significantly different (MWU, $P<0.05$, $n=16, 50$; Fig. S3A). In contrast, for the *A. m. capensis* clones, irrespective of increases in the amounts of HOB and 9-ODA, there were no significant differences in the production of individual MDG components between PCs and non-PCs (Fig. S3B). However, the total amounts

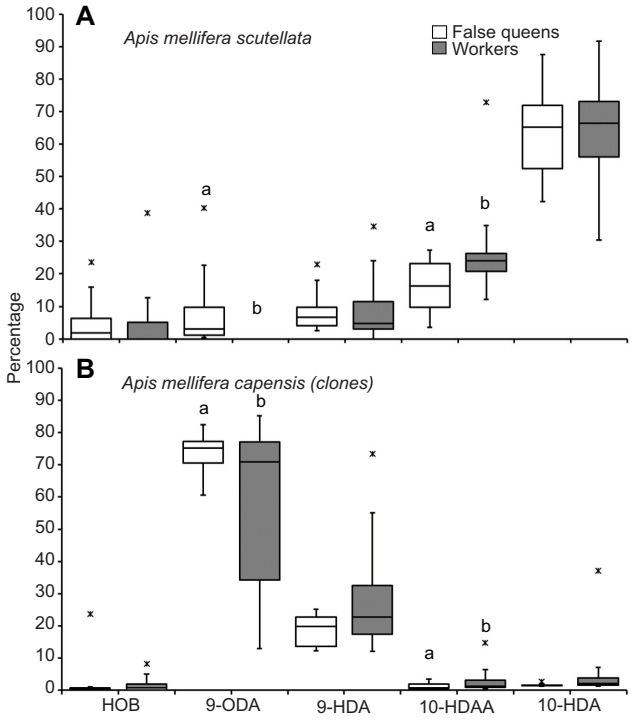

Fig. 1. Composition of mandibular gland components from false queens and workers of *A. m. scutellata* and *A. m. capensis*. Percentages of mandibular gland pheromones from cephalic extracts of (A) *Apis mellifera scutellata* and (B) *A. m. capensis* clones carrying Pseudo Queen® strips (open bars) and workers not carrying any strips (grey bars). Mandibular gland components are: HOB, *p*-hydroxybenzoate; 9-ODA, 9-oxo-2(*E*)-decenoic acid; 9-HDA, 9-hydroxy-2(*E*)-decenoic acid; 10-HDAA, 10-hydroxy-decanoic acid; and 10-HDA, 10-hydroxy-2(*E*)-decenoic acid. Middle lines shows the median, boxes represent first and third quartiles, whiskers are lower and upper bounds, outliers are represented by asterisks. Different letters on top of bars indicate significant differences between groups (MWU, $P<0.05$).

of 31.73±9.3 and 20.88±3.6 µg for PCs and non-PCs, respectively, were significantly different (MWU, $P<0.05$, $n=10, 50$; Fig. S3B).

Synthetic QMP versus clone extracts: pheromone production

Extracts from commercial Pseudo Queen® strips contained only five of the six known MDG components, with the worker substance 10-HDA not detectable (Table 1). The queen substance 9-ODA (38.66±2.87 µg) and its precursor 9-HDA (33.01±2.61 µg) are the main components from Pseudo Queen® extracts, whereas the clone extracts had 9-HDA (23.92±7.61 µg) as its main component (Table 1). Comparatively, the total components and the amounts of other components with the exception of 9-ODA (MWU, $P<0.05$, $n=5, 5$) and 10-HDA (MWU, $P<0.05$, $n=5, 5$) were not significantly different between the extracts from strips and those from the clone heads (Table 1).

Extracts from both Pseudo Queen® strips and clone heads were successfully applied to delivery septa. The septum absorbed all components (Fig. 2A) at the same rate with no differences except for the amounts of 9-HDA and the worker components 10-HDAA and 10-HDA (MWU, $P<0.05$, $n=5, 5$). Note that the latter was not originally present in the synthetic QMP extracts. Of interest are the amounts of queen substance 9-ODA that were absorbed by the septa (Fig. 2A). From the two extracts, 9.08±0.81 and 7.52±2.48 µg were absorbed from synthetic QMP PCs and clone extracts, respectively, making the amounts not significantly different from each other

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Table 1. Amounts and percentages of individual and total mandibular gland components extracted from strips of commercial Pseudo Queen® lures and single heads of *Apis mellifera capensis* clones

Mandibular gland component	Extract from Pseudo Queen® strips [µg (%)]	Extracts from clone heads [µg (%)]	*Z*	*P*
Methyl *p*-hydroxybenzoate (HOB)	6.15±0.73 (7.46)	6.47±3.01 (8.89)	0.522	>0.05
9-Oxo-2(*E*)-decenoic acid (9-ODA)	*38.66±2.87* (47.35)	*7.52±2.48* (27.53)	2.611	<0.05
4-Hydroxy-3-methoxyphenylethanol (HVA)	1.04±0.37 (1.31)	0.44±0.24 (0.63)	1.149	>0.05
9-Hydroxy-2-decenoic acid (9-HDA)	33.01±2.61 (40.38)	23.92±7.61 (39.01)	0.731	>0.05
10-Hydroxy-decanoic acid (10-HDAA)	2.82±0.24 (3.51)	4.17±2.00 (5.50)	0.104	>0.05
10-Hydroxy-2(*E*)-decenoic acid (10-HDA)	n.d.	*13.57±6.11* (18.44)	–2.611	<0.05
Total components	81.68±6.13	56.09±18.39	0.731	>0.05

Values in italics indicate there is a significant difference in the components between the Pseudo Queen® strips and clone head extracts (MWU, $P<0.05$). Values in brackets are mean percentages of each component in the extracts from five replicates. n.d., not determined. Data are means±s.e.m.

(MWU, $P>0.05$). In contrast, 9-HDA was absorbed more from clone extracts owing to its abundance in these extracts (Fig. 2A). On average, the total amount of components absorbed by the septa from Pseudo Queen® strips was 17.89±1.75 µg whereas that from the clone extracts was 56.09±18.39 µg. At the end of the experiments when delivery septa were checked, they contained 9-ODA and its precursor 9-HDA as major components (Fig. 2B), and all other MDG components were present.

All bees carrying pheromones from the Pseudo Queen® extracts ($n=19$) or clone extracts ($n=23$) produced MDG secretions that were queen-like containing HOB, 9-ODA and 9-HDA, with HVA not detected (Fig. 3). The following three components, HOB, 9-ODA and 9-HDA, were significantly higher in PCs than their trace amounts found in non-PC carriers (Fig. 3).

PCs carrying clone extracts had more 9-ODA and 10-HDA, whereas PCs carrying synthetic extracts had more HOB and 9-HDA (MWU, $P>0.05$, $n=19, 23$; Fig. 3A). Workers not carrying PCs were also different in the composition of their MDGs, with those from the clone extract group having more HOB, 9-ODA and 10-HDA and those from the Pseudo Queen® group having more 10-HDAA (Fig. 3B). Amounts of 9-ODA (0.75±0.30 µg; $t_{40}=-2.75$, $P>0.05$), 10-HDAA (0.38±0.02 µg; $t_{40}=-3.52$, $P>0.05$) and total components (2.91±1.33 µg; $t_{40}=-2.77$, $P>0.05$) were significantly higher in bees carrying *A. m. capensis* clone extracts compared with those carrying extracts from commercial Pseudo Queen® lures (MWU, $P>0.05$, $n=19, 23$; Fig. S4).

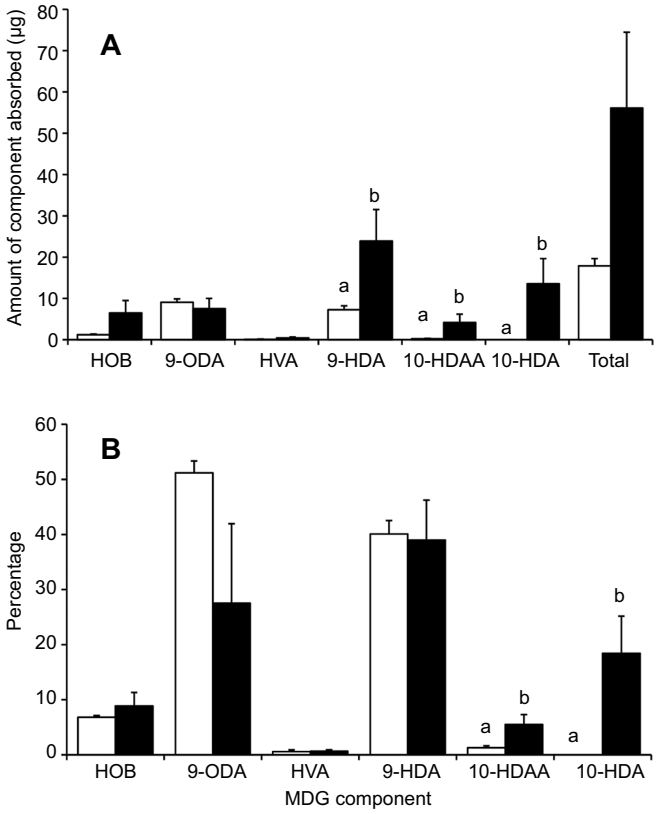

Fig. 2. Absorption and recovery of MDG components from pheromone delivery septum. (A) Amounts of mandibular gland (MDG) components absorbed by pheromone delivery septum and (B) proportions of the components recovered from the septum at the end of experiments. Open bars represent Pseudo Queen® extracts and closed bars represent extracts from clone heads. Individual mandibular gland components are as described in Fig. 1. Different letters on top of bars indicate significant differences between groups (MWU, $P<0.05$).

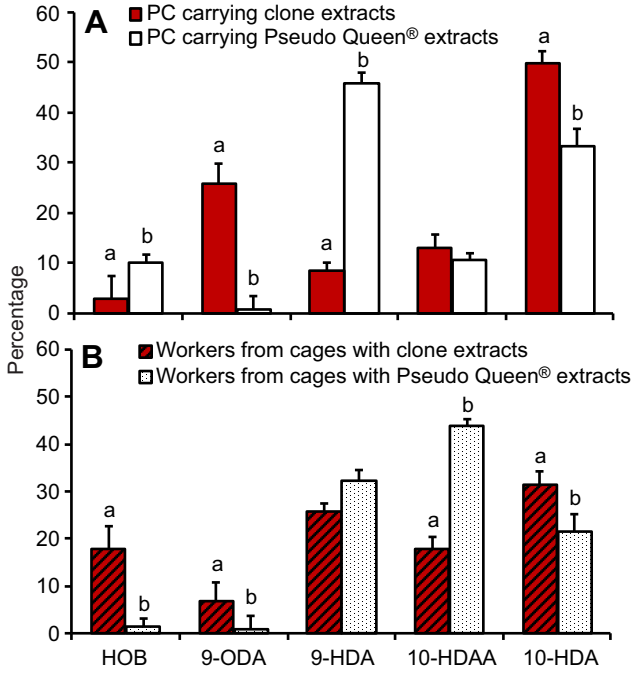

Fig. 3. Effects of carrying exogenous pheromones from clone extracts and Pseudo Queen extracts. (A) Percentages of mandibular gland pheromone components from cephalic extracts of *A. m. scutellata* pheromone carriers (PCs) carrying clone extracts and those carrying Pseudo Queen® extracts. (B) Percentages of mandibular gland pheromones from controls (non-PCs) reared in the same cages with clone extract PCs and with PCs carrying Pseudo Queen® extracts. Different letters on top of bars indicate significant differences in the components between groups (MWU, $P<0.05$).

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Ovarian activation

A total of 184 bees were dissected and their ovarian activation status assessed. All *A. m. capensis* clones had a spermatheca present, while it was absent in *A. m. scutellata* workers. Percentages of ovarian activation status for *A. m. capensis* clones and *A. m. scutellata* PCs are shown in Fig. 4A. Of the 10 *A. m. capensis* clone PCs, only three had fully activated ovaries (FD) while none of the 16 *A. m. scutellata* PCs had activated ovaries. A similar trend was also seen in the case of non-pheromone-carrying workers, indicating that synthetic QMP was effective in decreasing rates of ovarian activation in *A. m. capensis* workers and effective in suppressing ovarian activation in *A. m. scutellata* (Fisher's exact test, $P>0.05$).

When extracts from Pseudo Queen® and *A. m. capensis* clones were used on *A. m. scutellata* PCs, almost half of the PCs in both treatments did not activate their ovaries fully (FD) (Fig. 4B) but there was no significant difference between the two groups ($\chi^2=0.05$, d.f.=1, $P>0.05$). However, in the *A. m. capensis* extract control group, there were twice as many bees that had underdeveloped ovaries (UD) as those with developed ovaries (FD) (Fig. 4B).

DISCUSSION

Using cage experiments, we have shown that synthetic QMPs can elicit the production of MDG pheromones in worker bees and increase or initiate the production of queen-specific components such as 9-ODA and its precursor 9-HDA, thus transforming workers into false queens. This effect was seen not only in *A. m. scutellata*, whose workers do not produce significant amounts of 9-ODA (Zheng et al., 2010), but also in workers of *A. m. capensis* clones, which are known to produce queen-like pheromonal components from their MDG (Crewe and Velthuis, 1980). Recently, a study by Mumoki et al. (2018) demonstrated that honey bee queens can control the synthesis of MDG pheromones in reproductively active workers by blocking the production of alcohol dehydrogenase, the enzyme responsible for the oxidative reduction of 9-HDA into the queen substance (9-ODA). Thus, this explains why only false queens were able to produce queen-like signals in comparison with non-pheromone carriers. For all workers carrying synthetic pheromones, there was an increase in the total amount of pheromones produced by their MDGs, with *A. m. scutellata* PCs secretions becoming more queen-like and those in *A. m. capensis* worker clones increasing the production of the worker substance 10-HDA. We also observed that PCs from both subspecies elicited retinue formation within the experimental cages, similar to those of a live queen (Fig. S1) throughout the duration of the experiments. This attention may have resulted in increased trophallactic feeding by the attendant workers that may have triggered the production of queen-like pheromones and changes in pheromone profile, so that the PCs remained the dominant workers with the status of false queens. This observation is in agreement with those of Sakagami (1958) and earlier workers, who described the attraction of attendant workers to false queens.

Regarding the relative proportions, *A. m. scutellata* PCs still had more of the worker compounds 10-HDA and 10-HDAA in their MDGs. However, the proportions of 9-ODA and its precursor

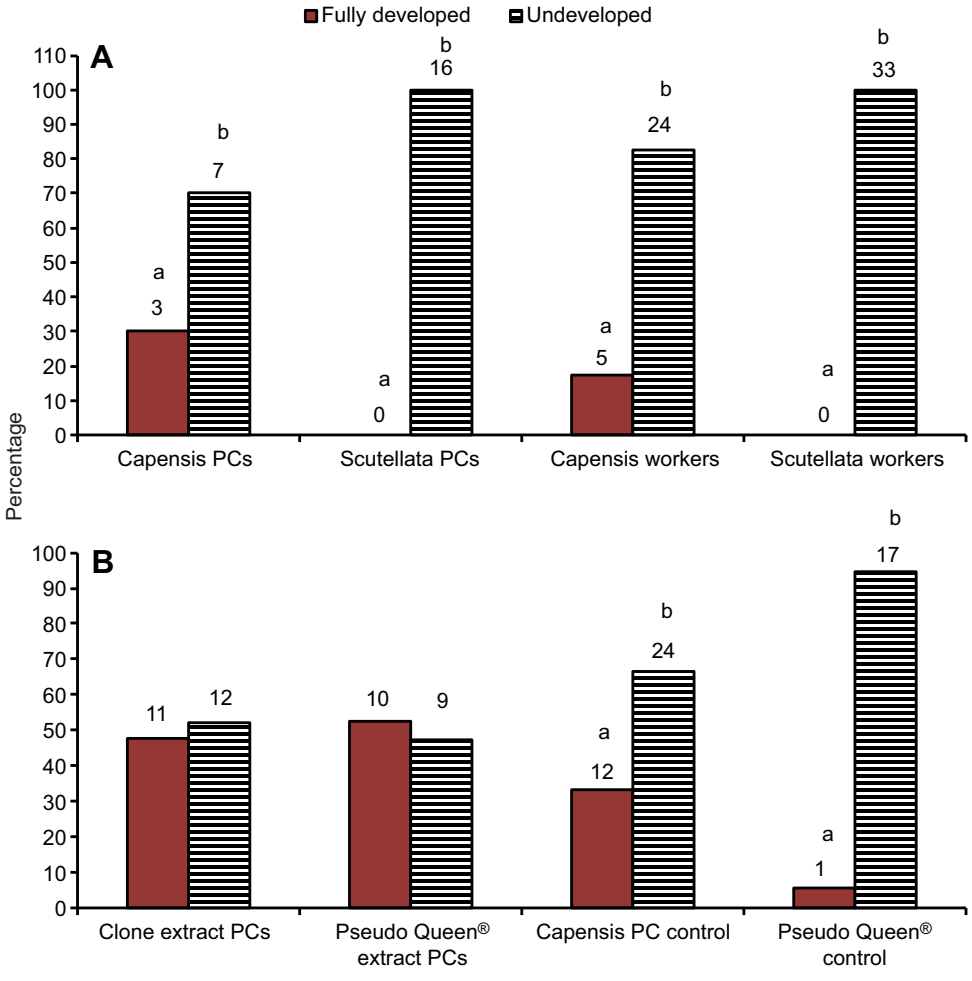

Fig. 4. Ovarian activation statuses of pheromone and non-pheromone carriers of *A. m. scutellata* and *A. m. capensis*. (A) Percentages of ovarian activation status in *A. m. capensis* clones carrying Pseudo Queen® (Capensis PCs), *A. m. scutellata* carrying Pseudo-Queen® (Scutellata PCs) and workers for both subspecies that were not carrying Pseudo Queen® lure. (B) Percentages of ovarian activation status of *A. m. scutellata* carrying clone extracts (Clone extract PCs), those carrying Pseudo Queen® extracts (Pseudo Queen® extract PCs) and those for the controls. Red filled bars denote bees that had fully activated their ovaries (FD, with ovarioles clearly visible), whereas striped bars represent bees with un-activated ovaries (UD, no evidence of developing ovarioles). Numbers represent the frequencies for each group of bees. Different letters indicate statistical differences between groups in A (Fisher's exact test) and B (χ^2 tests).

9-HDA increased to 7.70% and 8.44%, respectively, more than those reported in queen-right *A. m. scutellata* workers by Zheng et al. (2010). In contrast, the proportions of 9-ODA from *A. m. capensis* PCs remained within the ranges reported by Crewe and Velthuis (1980), showing a decrease in the proportion of 9-HDA, the precursor to the queen component.

Using a simple pheromone delivery mechanism, we were successful in impregnating GC septa with both synthetic pheromone components and natural pheromones extracted from *A. m. capensis* clone heads. Although the absorption efficiency of the septa was variable, 21% for the Pseudo Queen® and 60% for the clone heads (Fig. 2 and Table 1), the septa absorbed all the components present in these extracts, especially 9-ODA, with the total quantity of pheromones present at the same concentration for both extracts. These pheromones were present throughout the experimental trials (Fig. 2B), showing that the septa can be used as a clean and stable pheromone delivery source in bioassays, as was done for these pheromonal components. Thus, depending on the stability and classes of compounds impregnated into them, they could be used to explore the effects of other glandular sources of pheromones such as those of the tergal and Dufour's glands.

As with synthetic QMPs, PCs carrying pheromones extracted from synthetic lures and from *A. m. capensis* clones elicited the formation of retinues and had increased pheromone production, especially of the components HOB, 9-ODA and 9-HDA. However, PCs carrying clone extracts had more of the components that characterise queen pheromones. Although the synthetic QMPs were formulated based on profiles of European honey bee queens, the clone extracts were typical of an *A. m. capensis* blend of components that are more queen like than worker like, containing high amounts of the precursor to the queen substance (9-HDA) as well as the worker components 10-HDAA and 10-HDA. Hence, the *A. m. capensis* blend appeared to be more effective in the transformation of workers into false queens than synthetic QMPs. This was evident not only in the differences between amounts of components and total composition produced by the PCs, but also in that workers in the treatment with synthetic MDG extracts produced only trace amounts of HOB and 9-ODA, with their total MDG production being half of that produced by the group treated with the *A. m. capensis* clone extracts (Fig. S4).

Effects of synthetic QMPs on ovarian activation of *A. m. scutellata* and *A. m. capensis* PCs carrying strips of Pseudo Queen® were most evident in *A. m. capensis*, where 30% of PCs had fully developed ovaries while only 17% of non-PCs developed their ovaries. This was lower than the 37% of *A. m. capensis* clones reported to have activated ovaries under queenless conditions (Okosun et al., 2015). These results were surprising, as we had expected that as PCs in both subspecies increased pheromone production in their MDGs, they would activate their ovaries and become fully developed false queens. Thus, our results reaffirm findings in Backx et al. (2012) that both genetic and environmental manipulation affects ovary phenotypes in worker bees as shown by their treatments with synthetic QMPs. Backx and colleagues did not analyse pheromone production in their groups of bees and hence we do not know whether their treatment influenced pheromone production.

When different sources of QMPs (synthetic and those from clones) were carried by PCs, their ovaries were activated fully in both instances (Fig. 4B). This is an improvement from results obtained with only synthetic QMPs (Pseudo Queen®), where none of *A. m. scutellata* PCs had fully developed ovaries (Fig. 4A). Whilst most PCs fully activated their ovaries, approximately 30% of workers in the control group for the clone extracts had fully activated ovaries (Fig. 4B), an indication that clone extracts can be used to initiate ovarian activation in *A. m. scutellata*. Pheromonal dominance results in getting more food (Schäfer et al., 2006); this translates to more proteins for ovarian activation and pheromone production. Previous work (Altaye et al., 2010; Hoover et al., 2006; Pirk et al., 2010) has shown that diets do affect ovarian activation. The activation of ovaries in *A. m. scutellata* workers treated with MDG extracts from the clone was unexpected because it was assumed that their ovaries would be inhibited by the exogenous pheromone source. These differences in the efficacies between clone extracts and synthetic pheromones in regulating ovarian activation could result from the fact that PCs in both instances have different amounts of MDGs, with those carrying clone extracts having more pheromones in both quantity and quality, which could explain why *A. m. capensis* readily make pseudo-queens. However, the differential feeding of an individual that is a source of queen pheromones (PC) appears to have an effect on activation of its ovaries, thus enabling it to exercise dominance over other workers by inhibiting both their pheromone production and ovarian activation.

The fact that PCs activate their ovaries and become false queens complicates dominance relationships within the group rather than creating a situation in which worker reproduction would be effectively regulated, thus allowing easier re-queening of the colony in the event of queen loss.

In conclusion, we succeeded in creating mobile pheromone carriers. We also have shown that the use of exogenous pheromone supplements of either synthetic or natural origin does increase the production of queen-like pheromones in individual PCs, as well as elicit retinue behaviour around PCs. We have further demonstrated that QMPs can be use to initiate the production or transformation of workers into false queens in *A. m. scutellata* by initiating ovarian activation. This demonstrates that workers can be manipulated into becoming false queens irrespective of their genetic origin, further confirming earlier studies that attributed the making of false queens to the compositions of secretions from the mandibular gland pheromones, a mechanism that is used by honey bees when the queen is lost to initiate re-queening. Although, synthetic pheromones elicited retinue behaviour and the production of queen-like pheromones in workers, natural extracts performed better in increasing pheromone production and activation of ovaries.

Because Pseudo Queen® lures have QMP compositions based on the quantitative composition of European queen pheromones that are different from those of African honey bee queens, there is a need to explore how synthetic QMPs formulated in ratios similar to those of *A. m. scutellata* and *A. m. capensis* queens affect QMP production in workers under queenless conditions. The effect of these PCs as false queens on the regulatory dynamics of a colony of bees would need to be explored in more detail, particularly in relation to re-queening of queenless colonies. We have also shown the possibilities of making simple and robust pheromone delivery systems that can be further explored in both laboratory and field applications, especially where a clean and steady mobile pheromone delivery system is needed.

Acknowledgements
We thank O. O. Okosun for help with dissections.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.A.Y., R.M.C., C.W.P.; Methodology: A.A.Y., C.W.P.; Formal analysis: A.A.Y.; Investigation: A.A.Y.; Resources: A.A.Y., R.M.C., C.W.P.; Data

curation: A.A.Y.; Writing - original draft: A.A.Y.; Writing - review & editing: A.A.Y., R.M.C., C.W.P.; Project administration: R.M.C.; Funding acquisition: A.A.Y., R.M.C., C.W.P.

Funding
Funding was provided in part by the South African National Research Foundation (NRF) Research Career Advancement (RCA) Fellowship (grant no. 91491), DST/NRF SARCHI Chair M3B2 (grant no. 82770) to A.A.Y., and NRF incentive funding to A.A.Y., C.W.W.P. and R.M.C.

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.175505.supplemental

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Supplementary material

Table S1 Amounts of Mandibular gland components from thoraces washes of pheromone carriers and synthetic QMP (PseudoQueen®) strips extracted at the end of experiments

	Amount in µg						
Thorax Wash	**HOB**	**9-ODA**	**HVA**	**9-HDA**	**10-HDAA**	**10-HDA**	**Totals**
Pseudoqueen 1	0	0	0	0	0	0	**0**
Pseudoqueen 2	0	0	0	0	0	0	**0**
Pseudoqueen 3	0	0	0	0	0	0	**0**
Pseudoqueen 4	0	0	0	0	0.01	0	**0.01**
Pseudoqueen 5	0	0	0	0	0	0	**0**
Marked worker 1	0	0	0	0	0	0	**0**
Marked worker 2	0	0	0	0	0	0	**0**
Marked worker 3	0	0	0	0	0	0	**0**
Marked worker 4	0	0	0	0	0	0	**0**
Marked worker 5	0	0	0	0	0	0	**0**
Marked worker 6	0	0	0	0	0	0	**0**
Marked worker 7	0	0	0	0	0	0	**0**
Marked worker 8	0	0	0	0	0	0	**0**
Marked worker 9	0	0	0	0	0	0	**0**
Marked worker 10	0	0	0	0	0	0	**0**
Umarked worker 1	0	0	0	0	0	0	**0**
Umarked worker 2	0	0	0	0	0	0	**0**
Umarked worker 3	0	0	0	0	0	0	**0**
Umarked worker 4	0	0	0	0	0	0	**0**
Umarked worker 5	0	0	0	0	0	0	**0**
Umarked worker 6	0	0	0	0	0	0	**0**
Umarked worker 7	0	0	0	0	0	0	**0**
Umarked worker 8	0	0	0	0	0	0	**0**
Synthetic QMP strips extracted after experiments							
	HOB	**9-ODA**	**HVA**	**9-HDA**	**10-HDAA**	**10-HDA**	**Totals**
Strip 1	4.33	27.82	0	18.56	0.23	0.17	**51.12**
Strip 2	0.91	24.66	0	15.47	0.17	0.05	**41.27**
Strip 3	6.24	38.07	0	26.21	0.32	0.17	**71.02**
Strip 4	4.69	29.68	0	19.87	0.24	0.08	**54.56**
Strip 5	5.28	33.44	0	23.00	0.28	0.17	**62.16**

Supplementary figures

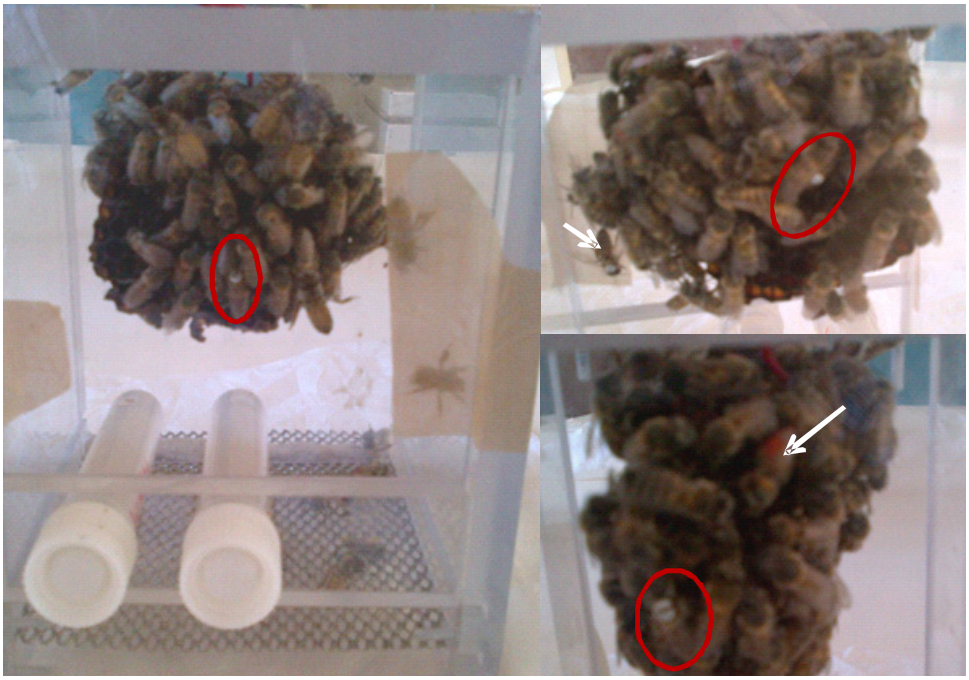

Fig. S1 Pheromone carriers (PCs) (red ellipse) surrounded by other bees and white arrows showing marked bees not carrying pheromones that attract no special attention from other bees in the hoarding cages.

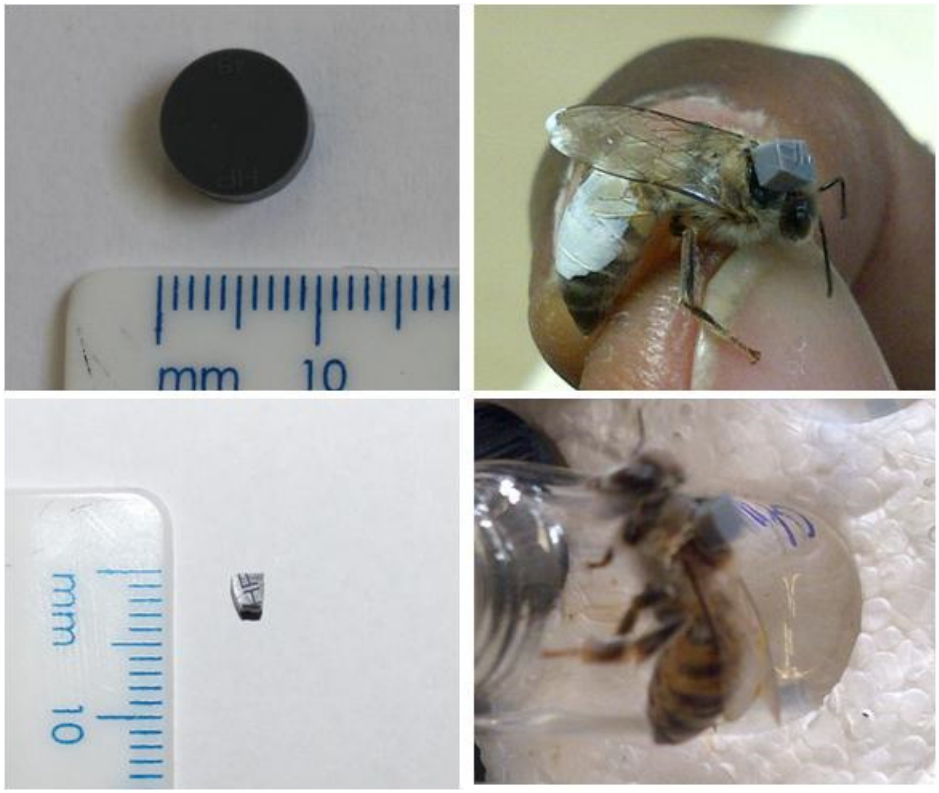

Fig. S2. Top and bottom left Gas chromatograph septa and cubes made from them. Top and bottom right, a worker bee with a septum cube on its thorax.

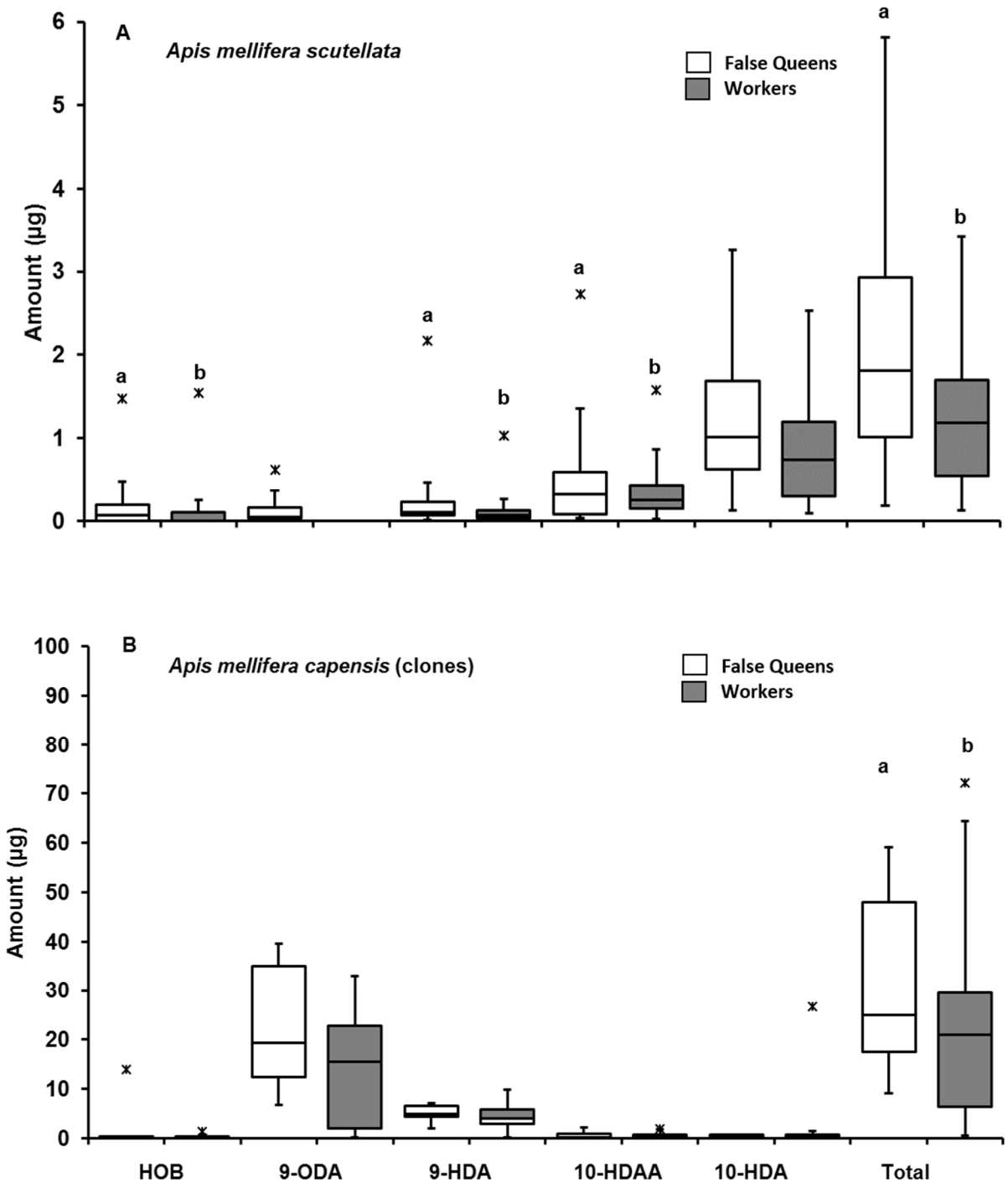

Fig. S3. Amounts of mandibular gland pheromones from cephalic extracts of *Apis mellifera scutellata* (A) *Apis mellifera capensis* clones (B), carrying Pseudo Queen® strips (open bars) and workers not carrying any strips (grey bars). Mandibular gland components are; HOB = *p*-hydroxybenzoate, 9-ODA = 9-oxo-2*(E)*-decenoic acid, 9-HDA = 9-hydroxy- 2*(E)*-decenoic acid, 10-HDAA = 10-hydroxy-decanoic acid and 10-HDA = 10-hydroxy-2*(E)*-decenoic acid. Middle lines shows the median, boxes represent 1st and 3rd quartiles, whiskers are lower and upper bounds, outliers are represented by asterisks. Different letters on top of bars indicates significant differences between groups (MWU, $p< 005$).

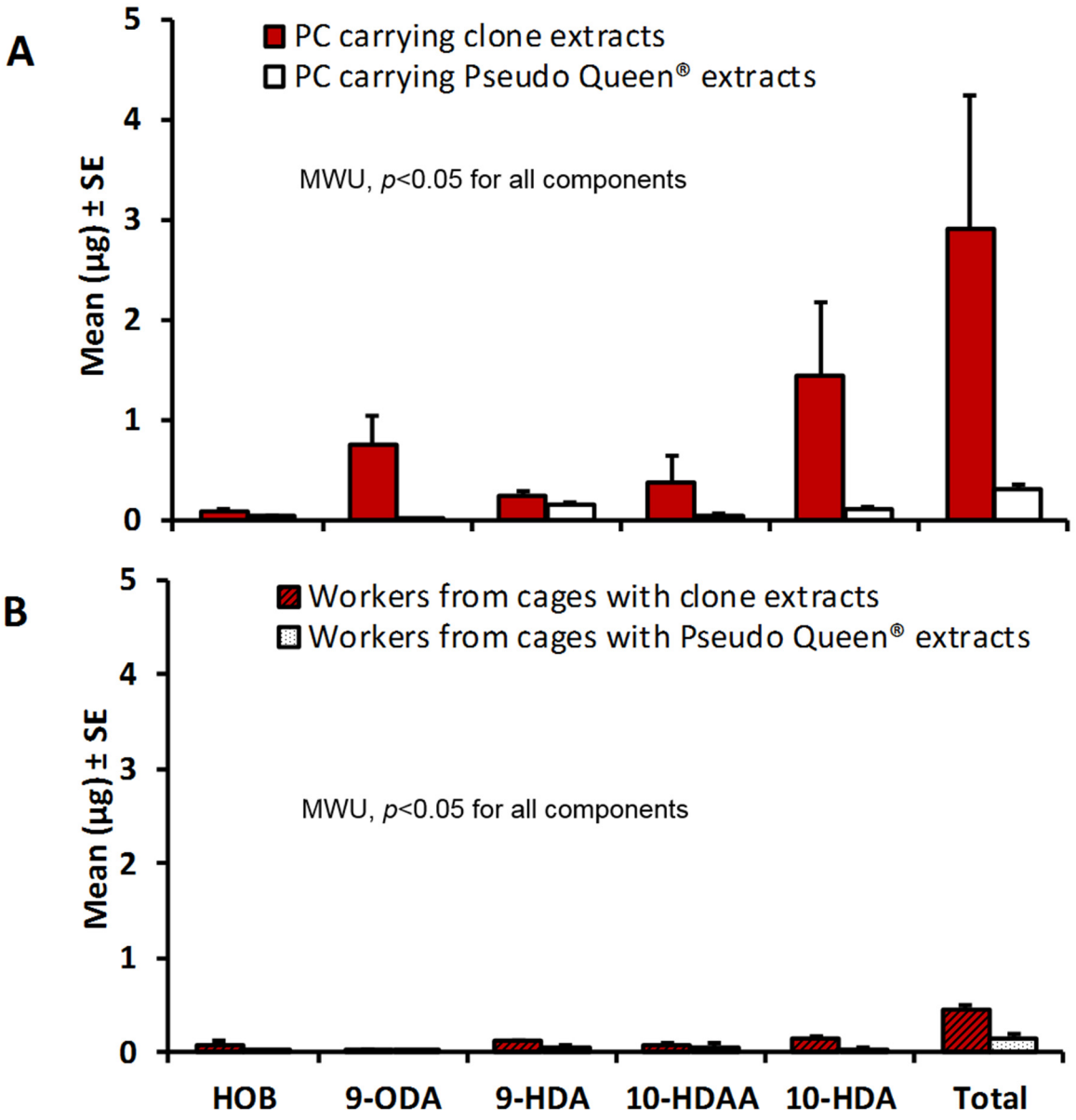

Fig. S4. Amounts of mandibular gland pheromone components from cephalic extracts *Apis mellifera scutellata* Pheromone carriers (PCs) carrying clone and those carrying Pseudo Queen ® extracts (A). Proportions of mandibular gland pheromones from controls (non-pheromone carriers) reared in the same cages with clone extract PCs and with PCs carrying Pseudo Queen® extracts are shown in B. Different letters on top of bars indicates significant differences in the components between groups (MWU, $p< 005$).