Control of mandibular gland pheromone synthesis by alternative splicing of the CP-2 transcription factor gemini in honeybees (Apis mellifera carnica)

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Original article

Control of mandibular gland pheromone synthesis by alternative splicing of the CP-2 transcription factor *gemini* in honeybees (*Apis mellifera carnica*)

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Abstract – The honeybee queen's mandibular gland pheromones (QMP) are essential for the suppression of worker reproduction. Worker ovary activation is regulated by alternative splicing of a CP2-transcription factor named *gemini*. Since workers with activated ovaries also produce QMP in their mandibular glands, we tested whether alternative splicing of *gemini* also controls mandibular gland pheromone biosynthesis in workers using RNA interference. Altering the splice pattern of *gemini* resulted in enhanced levels of the queen-specific components of the mandibular gland pheromone in queenless honeybee workers, suggesting that *gemini* functions as a pleiotropic regulatory switch influencing both ovary activation and resulting in QMP synthesis in workers. Because the QMP produced by these workers suppresses ovary activation in other workers, *gemini* seems to be a key regulatory gene affecting reproductive hierarchies among workers in queenless colonies.

RNA interference / ovary activation / worker reproduction / reproductive dominance / pheromone

1. INTRODUCTION

Eusocial insect colonies are maintained by numerous individuals whose activities need to be orchestrated in order to fulfil all necessary tasks and to overcome potential conflicts arising in every social community (LeConte and Hefetz 2008). Clearly, a fundamental conflict is that over reproduction within the colony. In honeybees (*Apis mellifera*) reproduction is normally monopolised by a single individual—the queen. By producing a pheromonal bouquet from her mandibular glands (queen mandibular gland pheromones: QMP) she controls many aspects of colony functioning including the inhibition of ovary development in her fellow workers (Hoover et al., 2003).

QMP comprises of a mix of fatty acids, esters, and aromatic compounds (Winston and Slessor 1998; Slessor et al. 2005). Five compounds have been singled out as particularly effective: 9-oxo-2(E)-decenoic acid (9-ODA, "the queen substance"; Butler et al., 1962), two enantiomers (cis-, trans) of 9-hydroxy-2-(E)-decenoic acid (9-HDA); and two aromatic compounds methyl-p-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (HVA) (Slessor et al. 1988, 1990). In contrast, the composition of the mandibular gland pheromones of workers differs from the bouquet produced by the queen (Callow et al. 1959; Plettner et al. 1996). Their mandibular

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gland secretions are dominated by hydroxy acids that have been functionalized at the last (ω) position such as 10-HDA and the corresponding saturated acid (10-HDAA) (Callow et al. 1959, Yusuf et al. 2015).

However, the composition of the mandibular gland bouquet is strongly dependent on the social context (Winston 1987; Crewe 1982; Plettner et al. 1997). Whenever the queen is lost, her inhibitory bouquet rapidly vanishes (Winston 1987; Slessor et al. 2005). Subsequently, a few workers activate their ovaries and develop into so called false queens (Sakagami 1958; Crewe and Velthuis, 1980; Hoover et al. 2003; Malka et al. 2007). In general, these unmated false queens produce haploid offsprings via parthenogenesis (arrhenotoky) which develop into males. They gain reproductive dominance in the colony as they are able to produce higher amounts of 9-ODA and 9-HDA than other workers (Malka et al. 2008; Moritz et al. 2004; Plettner et al. 1993). The development of such false queens is particularly swift in the Cape honeybee *Apis mellifera capensis* (Onions 1912; Ruttner et al. 1976; Crewe and Velthuis 1980) and even starts before the queen gets lost (Zheng et al. 2010). Uniquely, workers of this subspecies parthenogenetically produce female offspring (thelytoky) and hence the workers of a colony can rear queens from worker laid eggs to restore social stability (Moritz et al. 2011).

This exception to the rule of male haploid offspring of worker reproduction as well as the queen-like production of mandibular gland pheromones and the early onset of egg-laying (the laying worker syndrome) is thought to be controlled by a single genetic region (*th*-locus; Lattorff et al. 2005, 2007) with the homologue to the *Drosophila* transcription factor *gemini* (XM_001121158; XM_393898) as a candidate. Indeed, *gemini* showed interference with the alternative splicing of a single exon which was sufficient to initiate ovary activation in *A. mellifera carnica* (Jarosch et al. 2011). Although more recent studies have shown that *gemini* is not the ultimate genetic switch controlling the thelytoky syndrome (Oldroyd et al... 2016, Aumer et al. 2017), it is clearly tightly interwoven into the gene cascades that control worker reproduction. To resolve the role of *gemini* in the full set of traits in the laying worker syndrome we tested whether *gemini* also controls mandibular gland pheromone biosynthesis by knocking down the transcript responsible for ovary activation using RNAi.

2. MATERIALS AND METHODS

2.1. *gemini* knockdown by RNAi

mRNA depletion of *gemini* transcripts containing exon 5 was done as reported in Jarosch et al. (2011) briefly described below. Because transcripts missing exon 5 were not detected by qPCR in nonreproductive arrhenotokous workers (Jarosch et al. 2011), no silencing experiments were carried out to further reduce this already rare transcript. Instead, we targeted transcripts containing exon 5, which were highly abundant in all arrhenotokous workers, and substantially alter the ratio of unspliced vs. spliced transcripts and eventually activate the ovaries of *A. m. carnica* workers. Afterwards, two gemini-specific siRNA template sequences (78 bp) within the cassette exon 5 were selected using the siRNA target designer version 1.6 (Promega). The selected siRNA sequences were blasted against the honeybee genome to avoid potential off-target effects in genes other than *gemini*. None of the siRNAs shared sequence similarities longer than 14 nt (16- to 18-nt-long stretches of homology are suggested as the maximum acceptable length in RNAi studies per Ambion siRNA design guidelines). Furthermore, none of the genes with the highest sequence similarities was found twice when blasting both siRNAs individually. The two siRNAs obtained (see Jarosch et al. 2011) cover the 3′ and the 5′ regions of the template. Using this siRNA mixture decreased the chance of affecting unintended targets. siRNA was synthesised using the T7 Ribomax Express RNAi System (Promega) according to the manufacturer's instructions but with an extended incubation time of up to 2 h. The siRNA quality and quantity was assessed by capillary gel

electrophoresis and photometry. The knockdown of exon 5-containing transcripts caused an altered ratio of unspliced versus spliced transcript variants, and resulted in the activation of ovaries of honeybee workers treated in this way. A mixture of the two *gemini*- specific siRNA template sequences within the cassette exon 5 (78 bp) described above, and a scrambled siRNA was used as a negative control to account for potential off-target effects of the treatment (Jarosch et al. 2011).

2.2. Honeybees and siRNA feeding

Handling and feeding of newly emerged *A. m. carnica* workers (arrhenotokous) (1–2 days old) essentially followed the protocol from Jarosch et al. 2011. However, in this experiment, the *gemini* siRNAs and its associated scrambled siRNA were supplied with 2.5 ml 40% royal jelly (Naturprodukte Lehmbzke GbR; Mecklenburg-Vorpommern; Faulenrost) diluted in 50% sugar water. The bee groups, one per treatment, of about 35–40 individuals were kept in stainless steel cages (10 × 10 × 5.5 cm) provided with a small piece of comb at 32 °C. As honeybee workers under queenless conditions do activate their ovaries in less than 10 days (Velthius 1970; Ruttner and Hesse 1981). We assessed ovarian activation in the bees after 12 days in order to ensure that bees had enough time to do so. On the 13th day, bees were cooled down for decapitation. The head was directly transferred into 200 μl dichloromethane to extract the mandibular gland secretions. The extracts were stored at 4 °C until chemical analysis. The decapitated body was immediately frozen in liquid nitrogen and stored at – 80 °C until the dissection of the ovaries. Ovaries were classified as undeveloped with thin or slightly thickened ovarioles without oocytes. Ovaries were classified as developed when ovarioles were swollen and contained immature oocytes or mature oocytes. In this class of ovaries, the volume of the eggs exceeds the volume of the nutritive follicle (Hoover et al. 2003; Velthuis 1970). Significant differences between the sampled groups were analysed by chi-squared tests with p values corrected for multiple testing.

2.3. Chemical analysis of mandibular gland pheromones

The heads were removed from the dichloromethane solvent, 100-μl aliquots were evaporated to dryness under a nitrogen stream, re-dissolved in 10 μl internal standard solution containing octanoic acid and tetradecane (each at 0.4 mg/ml) in dichloromethane, and derivatised with 10 μl bis-(trimethylsily) trifluoroacetamide (BSTFA) for at least 4 h. One microliter of this solution was injected into an Agilent 6890 gas chromatograph equipped with a split–splitless inlet, a flame ionisation detector, and a dimethylpolysiloxane-coated fused silica capillary column (HP-1MS; 25 m × 0.2 mm, 0.33-μm film thickness). Samples were introduced by splitless injection. Helium was used as carrier gas (modified from Simon et al. 2001) with a flow rate of 1 ml/min in constant flow mode. The temperature program was set at 60 °C (1 min), 50 °C/min heating to 110 °C, 3 °C/min heating to 220 °C, and 220 °C (10 min). The inlet and flame ionisation detector were heated at 230 and 320 °C, respectively. Identification of HOB, 9-ODA, HVA, 9-HDA, 10-HDA, and 10-HDAA was based on retention times of synthetic compounds using the Agilent ChemStation® software version B.02.01. This suite of compounds are collectively referred to as the queen mandibular pheromone (QMP).The standard solution containing all six mandibular gland compounds was run at the beginning of chemical analysis and again after every 30th sample as a quality control check and to account for shifts in retention times. Tetradecane (internal standard) and the external standards were used to determine the relative mass ratios (Gehrke and Leimer 1971) of all tested compounds. For more details on the method see Yusuf et al. (2015).

2.4. Dissections

Dissected worker ovaries were classified as developed when the ovarioles were swollen and mature or immature oocytes visible or as undeveloped with thin or slightly thickened ovarioles without oocytes (modified after Hess 1942 and Pirk et al. 2010).

2.5. Statistical analysis

For statistical analyses the software STATISTICA 6.0 (Statsoft) was used. The variance in individual and total amounts of each of the measured QMP components between different treatments was analysed by Kruskal–Wallis ANOVAs and Median tests and subsequent multiple comparisons of mean ranks for all groups. To examine differences in the chemical profiles between the sample groups based on the relative proportion of every individual compound, a discriminant analysis was employed using all detected chemical compounds.

3. RESULTS

3.1. Ovary activation in individuals treated with exon 5-specific siRNA

As in Jarosch et al. (2011), honeybee workers treated with exon 5-specific siRNAs rapidly activated their ovaries and produced mature eggs. In this experiment, 3.6% of the siRNA-treated workers show no activation, while 96.4% show activated ovaries. This is a significant higher level of reproductive individuals in comparison to untreated workers ($p = 0.042$; 73.7% of workers with activated ovaries) and workers treated with unspecific siRNA ($p = 0.015$; 67.7% of workers with activated ovaries) (Figure 1).

3.2. Absolute amounts and the composition of mandibular gland compounds

The amount of the aromatic compound HVA was below the detection limit of the GC analyses performed (Table I). Notably it has been reported previously that HVA levels are undetectable or at very low levels even in virgin and newly inseminated queens (Slessor et al. 1990; Richard et al. 2007). Moreover, the total quantity of the remaining four compounds differed significantly between the three treatment groups (Kruskal–Wallis ANOVA; $H_{2,96} = 15.023$; $p = 0.0005$) (Table I). Whereas workers fed with 40% royal jelly only produced a total quantity of 1.43 ± 1.28 µg (mean ± S.D.), workers treated with scrambled siRNA emitted 2.02 ± 3.17 µg in total (multiple comparisons $z' = 0.367$; $p = 1.0$) (Table I). The *gemini* knockdown group produced a total quantity of 4.87 ± 5.79 µg which differs significantly from both, the RJ control (multiple comparisons $z' = 3.638$; $p < 0.001$) and the scrambled siRNA control group (multiple comparisons $z' = 3.142$; $p = 0.005$) (Table I). This means an increase in the total amounts of the mandibular gland components in the *gemini* knockdown individuals of 341% compared to RJ fed bees and 242% compared to workers treated with scrambled siRNA. In terms of proportions, only those of the queen substance 9-ODA (multiple comparisons $z' = 41.92$; $p = 0.00005$) and those of the worker component 10-HDAA (multiple comparisons $z' =$

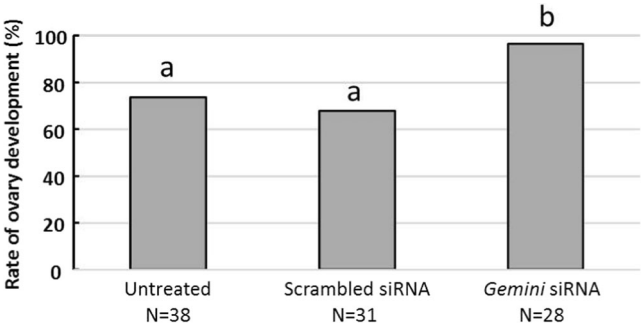

Figure 1. Ovary activation in *A. m. carnica* workers supplied with untreated diet ($n = 38$), scrambled siRNA ($n = 31$) or *gemini* -specific siRNA ($n = 28$). The knockdown by the combination of two transcript specific siRNAs leads to a significant increase of reproductive workers shown by different letters ($p \leq 0.05$, chi-squared tests with p values corrected for multiple testing).

Table I. Absolute amounts of mandibular gland pheromonal components (mean ± SD in microgrammes) in workers fed with royal jelly ($n = 38$), scrambled siRNA ($n = 31$) and *gemini*-specific siRNA ($n = 27$)

	Control	Scrambled siRNA	*Gemini* siRNA
Mandibular gland component	Mean ± SD (μg)	Mean ± SD (μg)	Mean ± SD (μg)
Methyl *p*-hydroxybenzoate (HOB)	0.02 ± 0.07	0.01 ± 0.05	0.00 ± 0.00
9-oxo-2*(E)*- decenoic acid (9-ODA)	0.14 ± 0.47	0.73 ± 2.40	2.39 ± 4.19
4-hydroxy-3-methoxyphenylethanol (HVA)	ND	ND	ND
9-hydroxy- 2- decenoic acid (9-HDA)	0.89 ± 0.66	1.10 ± 1.06	2.25 ± 2.38
10-hydroxy-decanoic acid (10-HDAA)	0.33 ± 0.42	0.11 ± 0.16	0.11 ± 0.08
10-hydroxy-2*(E)*- decenoic acid (10-HDA)	0.06 ± 0.08	0.07 ± 0.05	0.12 ± 0.05
Total components	1.43 ± 1.28	2.01 ± 3.17	4.87 ± 5.79

27.90; $p = 0.0005$) differ significantly between treatment groups. The following four components, HOB, 9-ODA, 9-HDA, 10-HDAA and 10-HDA were present in both the control and scrambled siRNA groups but absent in the secretions of individuals in which *gemini* was knocked down (Figure 2).

When examining the individual compounds, we observed two different patterns: first of all, components that were not affected and those that were increased by a knockdown of *gemini*. The pattern of unaltered synthesis can be seen in the second aromatic compound HOB ($H_{2,96} = 2.953$; $p = 0.228$) and in the second worker-specific compound 10-HDAA. Here, the siRNA-treated individuals do not differ compared to the untreated and scrambled control groups (respectively, $p = 0.2$ and $p = 0.6$). In contrast, the amounts of the queen-specific pheromones 9-ODA and 9-HDA as well as the worker-specific compound 10-HDA significantly increased in workers treated with exon 5-specific siRNA (Table I). In each case, workers unable to express the exon 5-containing transcript of *gemini* produced twice as much of the pheromonal components compared to the scrambled control.

In order to analyse whether different treatment groups had different pheromonal bouquets, we performed a discriminant analysis using all the components detected as variables. Indeed, there are significant differences in the relative proportions of the chemical compounds present in the mandibular glands across the three sample groups (Wilks's lambda: 0.6320, $F_{8,180} = 5.8008$, $p \leq 0.001$). Standardised coefficients for canonical variables for all components had an eigenvalue of 0.5148 and 0.0444 for roots 1 and 2 respectively. Mahalanobis distance (MD) of RNAi-treated worker chemical profiles is significantly different from both control group chemical profiles (All $MD \geq 1.98$ and $p \leq 0.001$). Workers in the scrambled RNA group were closer to the control (squared Mahalanobis distance = 1.3775) than those in the siRNA-treated group (squared Mahalanobis distance = 2.7859) clearly discriminating workers that were specifically treated to show a lower amount of transcripts that contain exon no. 5 of *gemini* (Figure 3).

4. DISCUSSION

Knockdown of exon 5-containing transcripts of *gemini* led to an activation of ovaries in honeybee workers kept in cages without a queen. Workers treated with siRNA blocking the transcriptional processing of certain *gemini* transcripts show a significantly higher number of reproductive workers compared to both control groups. This is in accordance with previous findings that linked *gemini* to reproductive dominance in honeybees (Jarosch et al. 2011). This ovary activation is accompanied by an altered MG pheromonal bouquet in queenless honeybee workers. The overall amount of QMP components increases and the bouquet of fatty acid-derived components become more queen-like. As the production of the aromatic mandibular gland compounds HOB and HVA were not affected by the knockdown of specific

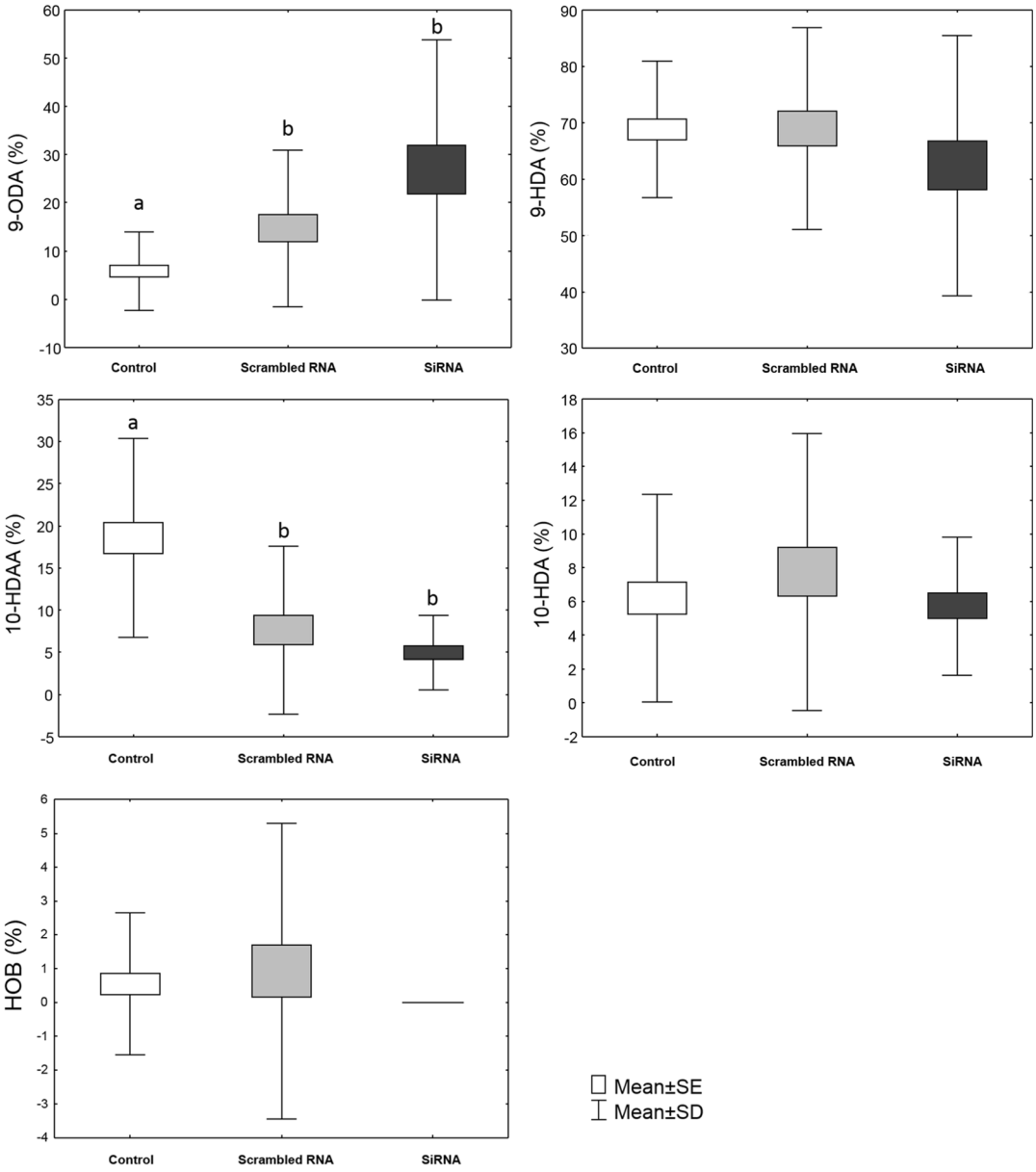

Figure 2. Composition of mandibular gland pheromonal components (mean ± SE in %) in workers fed with royal jelly ($n = 38$), scrambled siRNA ($n = 31$) and *gemini* -specific siRNA ($n = 27$). Different letters indicate significant differences between groups (Kruskal–Wallis ANOVA; multiple comparisons of mean ranks for all groups).

gemini transcripts, it seems that *gemini* acts on the biosynthetic pathway of the fatty acid-derived components.

The metabolism of these compounds takes place within the endoplasmatic reticulum (ER). Here, stearic-acid-Acyl-CoA is transformed into 17-hydroxy stearic-acid-Acyl-CoA (Malka et al. 2014). In *Drosophila melanogaster,* the homologous *gemini* locus (Chorsky and Belote 1994) has been shown to interact with the *transitional endoplasmic reticulum ATPase* (*TER94*; CG2331) (Giot et al. 2003). This protein has an

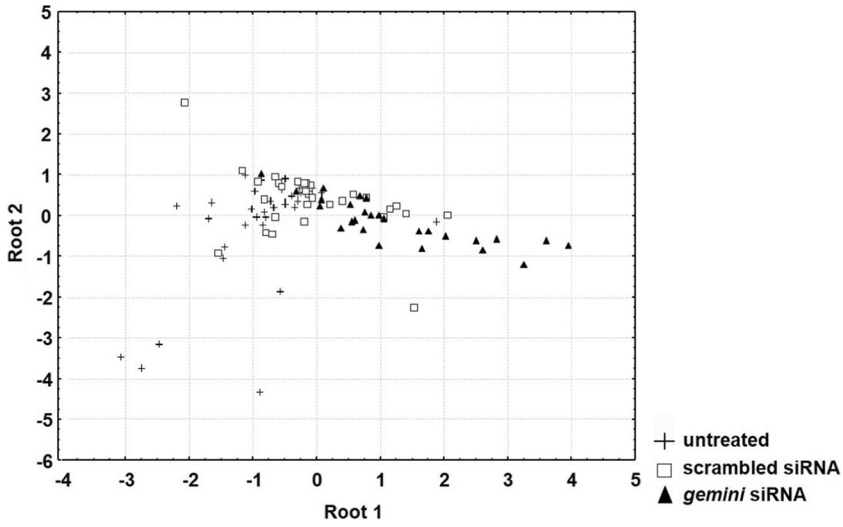

Figure 3. Position of honeybee worker individuals in a discriminant analysis performed on groups treated with either *gemini*-specific siRNA (▲), scrambled siRNA (□) or untreated (+). Each symbol represents a single individual.

ATP-binding and hydrolase activity and is involved in membrane fusion, as well as the organisation and the biogenesis of the ER. As the abundance of different *gemini* transcripts is adjusted in relation to a changed social context via alternative splicing (Jarosch et al. 2011), we may be observing an environmental stimulus giving rise to a physiological reaction. Upon queen loss, exon 5-deficient *gemini* transcripts are produced in greater abundance. Subsequently the ER biogenesis may be altered via interaction with *TER94* leading to a shift in the metabolism of long-chained fatty acids and thus to a change of the MG pheromone bouquet. As little is known about the biosynthesis of the two aromatic compounds HOB and HVA, they might simply be produced elsewhere in the cell and thus not influenced by *gemini*.

Such a conjunction between ovary activation and royal pheromone production on the removal of repression factors from the queen or brood has previously been shown to occur (Hepburn 1992; Simon et al. 2005; Malka et al. 2007). Although this coupling is expected to be genetically independent, we have demonstrated the existence of a pleiotropic gene that co-controls the expression of these two phenotypic traits upon queen loss which is essential for the establishment of reproductive dominance by individuals in groups of honeybee workers.

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Contributions AJP and RFAM concieved the idea, and AJP and AAY performed experiments and analysed and interpreted data. All authors participated in writing and revising the paper. All authors read and approved the final manuscript.

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Contrôle de la synthèse de la phéromone de la glande mandibulaire par épissage alternatif du facteur de transcription CP-2 *gemini* chez les abeilles mellifères (*Apis mellifera carnica*)

Interférence ARN / activation ovarienne / reproduction chez l'ouvrière / dominance reproductive / phéromone

Kontrolle der Synthese des Mandibeldrüsenpheromons durch alternatives Splicing des CP-2 Transkriptionsfaktors *gemini* in Hongbienen (*Apis mellifera carnica*)

RNA-Interferenz / Ovaraktivierung / Arbeiterinnenreproduktion / reproduktive Dominanz / Pheromon

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