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Short Communication

Reproduction of honeybee workers is regulated by epidermal growth factor receptor signaling ☆

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ABSTRACT

Eusocial insect societies display a remarkable reproductive division of labor between a single fertile queen and thousands of largely sterile workers. In most species, however, the workers retain the capacity to reproduce, particularly in queenless colonies where typically many workers lay eggs. As yet, the molecular determinants that initiate this shift in worker fertility are still poorly documented. By using RNA interference we here demonstrate that the knockdown of epidermal growth factor receptor, a gene which was previously shown to be involved in queen-worker caste differentiation, also induces reproduction in worker honeybees (*Apis mellifera*). These data show that worker fertility and queen-worker caste determination partly rely on the same gene regulatory networks, thereby providing a major breakthrough in our understanding of the molecular determinants of the social insects' spectacular reproductive division of labor.

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1. Introduction

The honeybees' reproductive division of labor between fertile queens and largely sterile workers has fascinated scientists for centuries. In fact, Darwin considered the near sterility of the workers a major evolutionary paradox and a 'special difficulty' to his theory of natural selection (Ratnieks et al., 2011). Since 2006 the honeybee genome became publically available (The Honeybee Genome Sequencing Consortium, 2006), which made the honeybee into a key model system to study the underlying molecular mechanisms of insect polyphenisms, such as those involved in queen-worker caste determination (Chan et al., 2006; Foret et al., 2012; Kamakura, 2011; Kucharski et al., 2008; Schwander et al., 2010).

Honeybee queen development is initiated early on in larval development. A specific diet, consisting of high amounts of royalactin, a monomeric form of major royal jelly protein 1, causes differentiation into queen-destined larvae (Kamakura, 2011). Downstream, caste determination relies on the insulin/insulin-like (de Azevedo and Hartfelder, 2008; Wheeler et al., 2006; Wolschin et al., 2011) target of rapamycin (TOR) (Patel et al., 2007) and epidermal growth factor receptor (EGFR) signaling pathways (Kamakura, 2011). In addition, the altered gene expression responsible for queen development has been shown to be caused partly by differential DNA methylation (Foret et al., 2012; Kucharski et al., 2008). These gene expression changes may result in an increased juvenile hormone (JH) titer in queens (Hartfelder and Engels, 1998; Rembold, 1987), and in a maturation of their ovaries. Adult queens can contain up to 200 ovarioles in contrast to the 3–26 ovarioles present in typical workers. Furthermore, alternative splicing of the *Gemini* transcription factor was also found to be involved in ovary activation (Jarosch et al., 2011).

The queen signals her presence to the colony using pheromones, which generally results in an inactivation of the ovaries of nearly all, 99.99%, of the workers (Kocher and Grozinger, 2011). Upon the irreversible loss of the queen, up to 30% of the worker bees activate their ovaries and start laying unfertilized, male-destined eggs (Ratnieks, 1993). Large-scale screenings of the genome (Linksvayer et al., 2009; Oxley et al., 2008), transcriptome (Cardoen et al., 2011; Grozinger et al., 2007; Thompson et al., 2006, 2008) and the proteome (Cardoen et al., 2011, 2012) delivered many candidate genes and proteins that might underlie this

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shift in the reproductive capacity of worker bees. A large-scale microarray study also revealed that there was significant overlap in the genes that were involved in regulating worker reproduction and queen-worker caste determination, including the epidermal growth factor receptor (Grozinger et al., 2007). Given this overlap and the fact that a recent study showed EGFR signaling to play a key role in queen-worker caste determination (Kamakura, 2011), we decided to test the involvement of EGFR signaling in honeybee worker reproduction. Additional evidence was provided by a microarray study comparing gene expression patterns in reproductive versus non-reproductive honeybees in queenless colonies (Cardoen et al., 2011) and showed that orthologues of three *Drosophila melanogaster* EGFR inhibitors (*Argos*, *Sprouty* and two paralogues of *Drosophila Cbl isoform A*) were upregulated in non-reproductive worker bees. Furthermore, a significant enrichment (ca. 8-fold) of genes categorized in the gene ontology function 'negative regulation of epidermal growth factor receptor activity' was observed in sterile workers (Cardoen et al., 2011), which implies that a decreased EGFR signaling likely induces worker sterility. Since down-regulation of EGFR, using RNA interference (RNAi) in queen-destined larvae resulted in a defective queen phenotype with undeveloped ovaries (Kamakura, 2011), we decided to test whether EGFR knock-down likewise inhibits ovary development in adult workers in a queenless environment.

2. Materials and methods

2.1. Honeybees and experimental procedures

For our experiments, we used *Apis mellifera carnica* honeybees that were reared at the experimental apiary of the Laboratory of Zoophysiology (Ghent, Belgium). Brood frames with emerging brood of six different honeybee colonies were collected on March 18th 2012 and incubated overnight at 34 °C and high relative humidity. The next morning, 300 newly emerged workers (max 24 h old) were collected from the six colonies and randomly mixed. For both the experimental and the control groups, 150 healthy honeybee workers were used and kept in three cages (approx. $10.5 \times 9 \times 7$ cm^3) containing 50 honeybees each. Every cage was equipped with a piece of beeswax, water, pollen paste and sugar dough containing 77% powdered sugar and 23% honey. All cages were incubated for 21 days at 34 °C and high relative humidity. Previous studies on honeybee ovary development were also successfully performed using cage experiments in order to mimic a queenless colony (Miller and Ratnieks, 2001), thereby obtaining workers with activated ovaries (Grozinger et al., 2007; Hoover et al., 2003, 2005). This approach also ensures that all treated bees can be analyzed without possible influences of untreated bees present in a natural queenless hive (Katzav-Gozansky et al., 2006).

Prior to caging these newly emerged worker bees were injected with siRNA targeting either the target EGFR gene or the non-target control gene GFP (a non-honeybees gene). Three different siRNA sequences to knock down EGFR and three sequences for GFP were mixed (Table S1). All fragments were purchased at Sigma–Aldrich (France). All siRNA fragments were dissolved in insect saline buffer (150 mM NaCl, 10 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid), vortexed, then shortly sonicated and spun down. An overall amount of 60 pmol of siRNA mix (dissolved in a volume of 2 μl) was injected in the back of the thorax with a 33 gauge needle. For each target one needle was used. The injection spot was sealed using melted synthetic wax (Syncera) at approximately 60 °C. Differences in survival among treatments were assessed using a binomial mixed model, in which cage was coded as a random factor. This was done using function glmer in package lme4 in R 2.15.

2.2. qRT-PCR validation of knockdown

Seven days post injection, 5 honeybees of each cage (i.e. 15 per experimental group) were randomly sampled, snap-frozen in liquid nitrogen and stored at −80 °C until further analysis. Total RNA was extracted from individual whole honeybees with the RNeasy lipid tissue mini kit (Qiagen) following the manufacturer's guidelines. Reverse transcription was performed *in duplo* starting with 5 μg RNA of each sample using Oligo (dT) primers (0.5 μg/μL) and was carried out according to the RevertAid H Minus First strand cDNA Synthesis kit protocol (Fermentas). Concentration and sample quality after each protocol was determined using Nanodrop 2000 (Thermo Fisher Scientific). Quantitative Reverse Transcriptase PCR (qRT-PCR) was performed using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen) and CFX96 Real-Time PCR Detection System (Biorad). Primers were developed with Primer Express 2.0 (Applied Biosystems) and validated by standard and melt curve protocols. The three reference genes (Table S1) used in this study were selected according to Cardoen et al. (2011), who studied the same phenotype.

Normalized target gene expression levels were calculated for every bee, using the comparative Ct method and the geometric mean expression level of the three best (most stably expressed) reference genes: GB10903 (ribosomal protein L32), GB16844 (elongation factor 1-alpha) and GB12747 (eukaryotic translation initiation factor 3 subunit C) (Kucharski et al., 2008). Log2 transformed relative expressed levels were statistically compared using a general linear mixed model, in which cage was included as a random factor. This was done using the statistical software R 2.15, using function lmer in package lme4, and assessing significance using the pvals.fnc function in package language R.

2.3. Assessment and comparison of worker ovary development

Three weeks post injection, dissection of the remaining workers was performed. Bee abdomens were dissected and the ovary activation was scored. Ovary activation was scored on a scale from 0 to 3, based on the scale described in Lin et al. (1999), with score 0 being used for ovaries that were undeveloped and in which no oocytes could be distinguished, a scores of 1, 2 or 3 being awarded when ovarioles contained visible round oocytes, sausage-shaped oocytes, or at least one fully developed egg, respectively (Fig. 1B). Worker ovary development was compared among the treatment groups using an ordered mixed logit model in which cage was coded as a random factor. This was done using function clmm in package ordinal in R 2.15.

3. Results

Quantitative real-time PCR revealed that EGFR knock-down was successful in bees sampled 7 days post injection (2.6-fold down-regulation, $p = 2.2E{-}6$, compared to green fluorescent protein (GFP) siRNA-injected control bees) (Fig. 1A; Table S2). In addition, mortality was not significantly different between the treatment groups ($p > 0.05$ for all comparisons, binomial mixed model, Table S3). Importantly, bees injected with siRNA targeting EGFR showed significantly reduced levels of workers ovary activation compared to control bees injected with siRNA targeting the non-honeybee gene GFP (Fig. 1B; Table S4; ordered mixed logit model, $p = 0.005$). This provides supporting experimental proof that EGFR signaling is involved in regulating reproduction in honeybee workers.

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