Short Communication

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

Ellen M. Formesyn a,1, Dries Cardoen b,1, Ulrich R. Ernst c, Ellen L. Danneels a, Matthias Van Vaerenbergh a, Dieter De Koker a,⇑2, Peter Verleyen c, Tom Wenseleers b, Liliane Schoofs c, Dirk C. de Graaf a,b,⇑

a Laboratory of Zoophysiology, Ghent University, B-9000 Ghent, Belgium
b Laboratory of Socio-ecology and Social Evolution, KU Leuven, B-3000 Leuven, Belgium
c Research Group of Functional Genomics and Proteomics, KU Leuven, B-3000 Leuven, Belgium

Keywords:
Reproductive division
Epidermal growth factor receptor
Fertility
Hymenoptera
Honeybee
Apis mellifera

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
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, we present in a natural queenless hive (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 × 9 × 7 cm²) 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-honeybee 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 CaCl₂, 2 mM MgCl₂, 10 mM 2-(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 (Syncrea) 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 or 2 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.
compared among treatments and with cage being included as a random factor. With siRNA targeting EGFR showed significantly reduced levels of workers ovary activation compared to control bees injected with siRNA targeting thenon-honeybee gene calculated using a general linear mixed model in which cage was coded as a random factor. EGFR knock-down induces near-sterility in honeybee workers (B). Bees injected with siRNA targeting the EGFR gene. EGFR was 2.6-fold down-regulated (Fig. 1.

4. Discussion

EGF signaling has been very well investigated in Drosophila due to its involvement in a wide array of physiological processes (Weyers et al., 2011). In honeybees, EGFR has previously been shown to be a key element in queen development (Kamakura, 2011). Our data are the first to provide supporting evidence, based on RNA interference knockdown, that EGF signaling is also involved in regulating reproduction in honeybee workers. In particular, down-regulation of EGFR resulted in a situation in which workers could no longer activate their ovaries in a queenless environment. This is interesting, because even though in former microarray (Cardoen et al., 2011; Grozinger et al., 2007) and proteomic analyses (Cardoen et al., 2011, 2012) neither EGFR or its likely ligand, gurken, were differentially expressed between reproductive and nonreproductive workers, and the EGFR inhibitors Argos, Sprouty and Chl were all upregulated in sterile workers (Cardoen et al., 2011). Furthermore, the genes upregulated among sterile workers in queenless colonies were ca. 4-fold enriched with the gene ontology function ‘negative regulation of epidermal growth factor receptor signaling pathway’ (p = 0.01), ca. 8-fold enriched for the GO term ‘negative regulation of epidermal growth factor receptor activity’ (p = 0.02) and ca. 2-fold enriched for the GO term ‘transmembrane receptor protein tyrosine kinase signaling pathway’ (p = 0.02) (Cardoen et al., 2011). This implies that down-regulation of the EGF signaling pathway likely plays a key role in the suppression of worker ovary activation, whereas conversely, its upregulation plays an important role in initiating worker egg-laying in queenless colonies. In addition, downstream factors such as Raf kinase and suppressor of cytokine signaling 5 (SOCS-5, which causes negative regulation of EGFR signaling), were also upregulated among sterile workers (Cardoen et al., 2011). All these data strongly support that EGFR signaling plays a key role not only in queen determination in the larval stage (Kamakura, 2011), but also in regulating reproduction in adult honeybee workers. This is consistent with the fact that EGF signaling is known to be involved in both embryonic gonad development (Weyers et al., 2011) and oocyte maturation in adult individuals in other invertebrate model organisms (Poulton and Deng, 2006) and that EGFR signaling is also involved in the negative regulation of apoptosis (Parker, 2006). Indeed, both worker ovary development in the larval stage and the suppression of worker ovary development in queenright colonies has been shown to involve specific patterns of programmed cell death (Cardoen et al., 2012; Tanaka and Hartfelder, 2004; Capella and Hartfelder, 1998). As it was demonstrated earlier that honeybee ovaries are highly resistant to taking up long or short dsRNAs (Jarosch and Moritz, 2011) it seems quite probable that the regulation of reproduction via the EGF signaling occurs indirectly and via extravarian mechanisms.

Acknowledgments

We acknowledge FWO-Flanders (G041708N and G062811N) and the KU Leuven Research Foundation (GOA 2010/14) for financial support. U.R.E. and M.V.V. were funded by IWT-Flanders.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2013.12.001.

References


Oxley, P.R., Thompson, G.J., Oldroyd, B.P., 2008. Four quantitative trait loci that influence worker sterility in the honeybee (Apis mellifera). Genetics 179, 1337–1343.


