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# MicroRNA signatures characterizing caste-independent ovarian activity in queen and worker honeybees (*Apis mellifera* L.)

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## Abstract

**Queen and worker honeybees differ profoundly in reproductive capacity. The queen of this complex society, with 200 highly active ovarioles in each ovary, is the fertile caste, whereas the workers have approximately 20 ovarioles as a result of receiving a different diet during larval development. In a regular queenright colony, the workers have inactive ovaries and do not reproduce. However, if the queen is sensed to be absent, some of the workers activate their ovaries, producing viable haploid eggs that develop into males. Here, a deep-sequenced ovary transcriptome library of reproductive workers was used as supporting data to assess the dynamic expression of the regulatory molecules and microRNAs (miRNAs) of reproductive and nonreproductive honeybee females. In this library, most of the differentially expressed miRNAs are related to ovary physiology or oogenesis. When we quantified the dynamic expression of 19 miRNAs in the active and inactive worker ovaries and compared their expression in the ovaries of virgin and mated queens, we noted that some miRNAs (*miR-1, miR-31a, miR-13b, miR-125, let-7 RNA, miR-100, miR-276, miR-12, miR-263a, miR-306, miR-317, miR-92a* and *miR-9a*) could be used to identify reproductive and nonreproductive statuses independent of caste. Furthermore, integrative gene networks suggested that some candidate miRNAs function in the process of ovary activation in worker bees.**

**Keywords: microRNA, transcriptome, ovary, plasticity, development, honeybee.**

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## Introduction

Honeybee (*Apis mellifera*) female ontogenies are governed by identical genomes. However, differences in the quantities of proteins and carbohydrates offered to young larvae trigger specific developmental pathways, producing highly specialized female phenotypes: queens and workers. The resultant dimorphism can be recognized in the entire organism, and the reproductive structures are markedly affected. The queens are equipped with huge ovaries and a well-developed spermatheca, an organ capable of storing millions of spermatozoa from multiple mates to ensure an oviposition rate of approximately 2000 eggs per day for several years under ideal circumstances. By contrast, the workers develop profoundly modified reproductive organs, with small ovaries and vestigial spermathecae. Additionally, workers do not mate and are known as the sterile members of this sophisticated society (Free, 1987). Chemical cues synthesized by the sole queen (Hoover *et al.*, 2003) and the larvae (Oldroyd *et al.*, 2001; Maisonnasse *et al.*, 2010) in a wild-type queenright colony maintain the nonreproductive status of thousands of workers.

However, worker sterility is reversible under queenless conditions, in which some workers activate their ovaries to produce oocytes that are laid as unfertilized eggs that develop into drones (for review, see Free, 1987).

The genetic mechanisms underlying the reproductive plasticity observed in adult workers are only partially understood from a coding gene perspective. Most studies have focused on gene expression profiles to identify factors that control worker sterility. Comparisons have been performed in various biological contexts ranging from gene-by-gene analyses (Thompson *et al*., 2007; Koywiwattrakul & Sittipraneed, 2009; Vergoz *et al*., 2012) to large-scale genetic studies (Thompson *et al*., 2006; Grozinger *et al*., 2007; Oxley *et al*., 2008; Thompson *et al*., 2008; Cardoen *et al*., 2011; Niu *et al*., 2014). In addition, the aforementioned studies collectively indicate the existence of an environmentally responsive regulatory network by which workers switch their ovaries 'on' or 'off'. Thus, it is possible that other genetic factors, such as microRNAs (miRNAs), play roles in a potentially complex network that regulates both ovary physiology and reproductive status.

miRNAs are small (19 to 24 nucleotides) nonprotein-coding transcripts that act as inhibitors of the expression of targeted eukaryotic coding genes (reviewed by Bartel, 2009). miRNAs have emerged as key elements in the regulation of essentially all biological processes in animals and plants (Marco *et al*., 2013). For example, miRNAs are related to development (Zhang *et al*., 2012), cell proliferation, tissue size (Nolo *et al*., 2006) and alternative phenotypes (Legeai *et al*., 2010). The functional importance of miRNAs in insect ovaries has been determined for holometabolous (Reich *et al*., 2009; Poulton *et al*., 2011) and hemimetabolous (Cristino *et al*., 2011) species. Given that the previously studied insects live in nonsocial contexts, the investigation of miRNAs in *A. mellifera* undergoing ovary activation would provide a valuable opportunity to understand the molecular pathways associated with reproduction in socially regulated environments.

Here, we present the expression profile of miRNAs from a small RNA deep sequencing library (RNA-Seq) of honeybee worker activated ovaries. We selected 19 miRNAs, out of 138 expressed in the library, with which to perform a comparative analysis using honeybee ovaries in different activation conditions: workers with activated (AW) or inactive (IW) ovaries, as well as mated (MQ) or virgin (VQ) queens. The expression of various miRNAs (*let-7RNA, miR-1, miR-9a, miR-13b, miR-31a, miR-92b, miR-100, miR-125, miR-276, miR-306* and *miR-317*) is characteristic of ovary status and distinguishes between the activated (found in AW and MQ) or inactive (found in IW and VQ) ovaries, suggesting that miRNAs expressed in the ovaries are caste independent. We reconstructed a putative integrative network (miRNA : mRNA) to evaluate the environmental perturbation caused by the absence of the queen using quantitative analysis of miRNA expression and prediction of their targets (mRNAs) in the activated and inactive worker ovaries. This reliable tool (see Joshi *et al*., 2015) allowed us to suggest roles for these regulatory molecules in ovary activation in honeybees.

## Results

### *Library description and identification of the expressed miRNAs*

We analysed a small RNA-Seq library obtained from the activated ovaries of honeybee workers as a first approach to evaluate the expression of specific regulatory molecules important for ovary function, the miRNAs. The sequencing generated a total of 88 942 714 reads (raw data). Approximately 34 273 775 reads mapped to unique regions of the *A. mellifera* genome, and 1.5 million reads mapped to known honeybee mature miRNAs (miRBase 19). Honeybee worker ovaries express 138 known miRNAs (Supporting Information Table S1). The 15 most highly expressed miRNAs in the activated ovary library are presented in (Fig. 1).

### *miRNA expression in activated and inactive ovaries*

Based on the library analysis, on mapping the sequences to the honeybee genome and on read counts, we selected 19 miRNAs. For validation, we take into account proofed function in other animals, as well as expression levels in activated and inactive ovaries from virgin and mated queens, and from workers obtained from queenright and queenless colonies. Our data demonstrate that the selected miRNAs were (similarly or differently) expressed in all of the tested conditions.

Amongst the tested miRNAs, *miR-1, miR-31a, miR-13b, let-7, miR-125, miR-100* and *miR-276* (Fig. 2A–G) were up-regulated in the inactive ovaries. We could also include *miR-12* in this group, given that it exhibited the same modulation in the workers and queens. However, when the workers with activated and inactive ovaries were compared, no significant differences were noted for this miRNA. This group contains genes with known functions in honeybees. *miRNA-1* has been described as essential for muscle development (Chen *et al*., 2006; Koutsoulidou *et al*., 2011) and *miR-31a* exhibits increased expression in nurse bees (Liu *et al*., 2012) and reduced expression with the ageing process (result not shown).

The remaining miRNAs were upregulated in the activated ovaries. This group is composed of *miR-306,* which is the most expressed in the library, *miR-92b* and

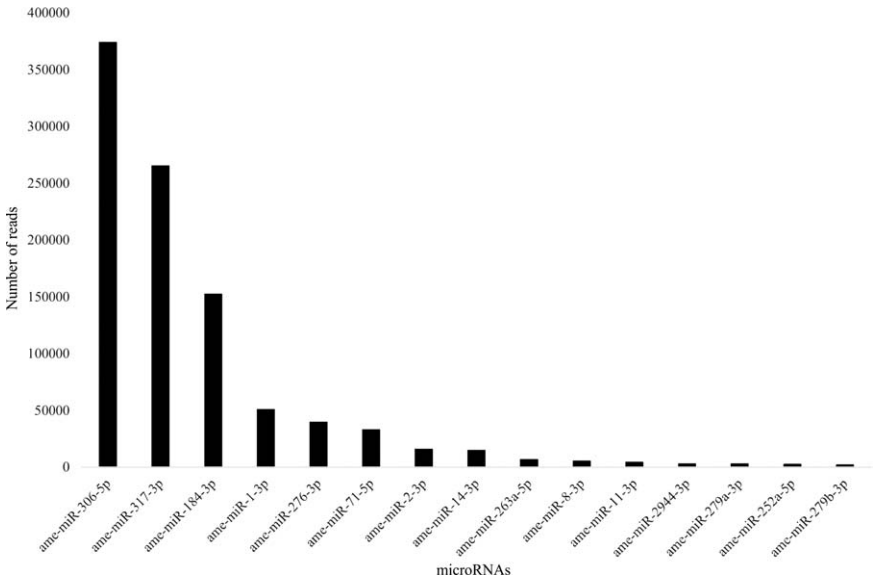


**Figure 1.** The 15 microRNAs (miRNAs) with the highest expression in the RNA-Seq library constructed from the activated ovaries of the worker honeybees.

*miR-9a* (Fig. 2J, L, M). The group also includes *miR-317,* the expression of which was only statistically significant in the ovaries of queens, MQ and VQ (Fig. 2K). This group of miRNAs showed increased expression in activated ovaries compared with inactive ovaries when analysed by quantitative PCR (qPCR). Similar to our observations here in honeybees, *miR-306* was also detected at high levels in *Drosophila* oocytes (Chen *et al*., 2014). *miRNA-9a*, the most strongly caste-biased miRNA amongst *A. mellifera* miRNAs (Weaver *et al*., 2007), exhibited increased expression in activated ovaries compared with inactive ovaries. However, expression was independent of the origin of samples, worker or queen (Fig. 2M), similar to *miR-92b* (Fig. 2L).

These results were used to investigate whether the expression levels of the miRNAs in the inactive (Fig. 2A–I) and activated ovaries (Fig. 2J–M) characterize the nonreproductive and reproductive condition of the ovaries in honeybees, independently of caste.

The library consists of members of the *miR-2 cluster, miR-2-1, miR-2-2, miR-2-3, miR-2b, miR-13a, miR-13b* and *miR-71.* This miRNA family is widely identified in invertebrates and is the largest family of microRNAs in *Drosophila melanogaster*. Interestingly, *miR-2-1, miR-2-2* and *miR-2-3,* which were assessed as *miR-2* and are amongst the most expressed miRNAs in the library (Fig. 1), did not characterize activated or inactive ovaries (Fig. 2O). These miRNAs were so homogenously expressed that they were used as a normalizer together with *miR-184* and *U5* (spliceosomal small nuclear RNA) in the qPCR reactions. However, a member of the same family, *miR-13b,* which is up-regulated in forager bees (Liu *et al*., 2012), characterizes inactive ovaries in the workers and the virgin queens (Fig. 2C). The other members of the *miR-2* family, *miR-13a* and *miR-71,* were equally expressed in all of the tested conditions (Fig. 2P, S). In *Blattella germanica*, three members of this cluster, *miR-13a, miR-13b* and *miR-71,* are closely related to metamorphosis (Lozano *et al*., 2015).

Several analysed miRNAs (*miR-2, miR-3720, miR-13a, miR-11, miR-184* and *miR-71*) exhibited stable expression, with no difference in the expression levels amongst AW, IW, MQ and VQ. Thus, these miRNAs were used as normalizers in the qPCR reactions (*miR-2* and *miR-184*), as mentioned above.

### *Predictive regulatory network*

To infer information on regulatory interactions regarding the regulatory function of miRNAs in the transition from nonreproductive to reproductive status in honeybee workers, we searched for miRNA targets amongst the miRNAs that were differentially expressed in the qPCR experiments (ie *miR-9a, miR-31a, miR-263a, miR-13b, miR-1, miR-276, miR-306 and let-7*). As an additional experimental validation, aiming to infer regulatory interactions (miRNA : mRNA), we searched for seed sequences in the 3′ untranslated regions (3′UTRs) of the differentially expressed mRNAs and honeybee proteins described by Cardoen *et al*. (2012) as having roles or involvement in activated and inactive worker ovaries. A total of 143 out of 153 of the recovered 3′UTRs presented one or more predicted sites for our differentially expressed worker ovary miRNAs (Table S3). For the reconstruction of the predictive miRNA-target regulatory network, we selected those miRNAs that best met the prerequisites of the program used, RNAHYBRID (Kruger & Rehmsmeier, 2006; Experimental procedures). Two miRNAs up-regulated in activated ovaries (*miR-306* and *miR-9a*) and six miRNAs up-regulated in inactive ovaries

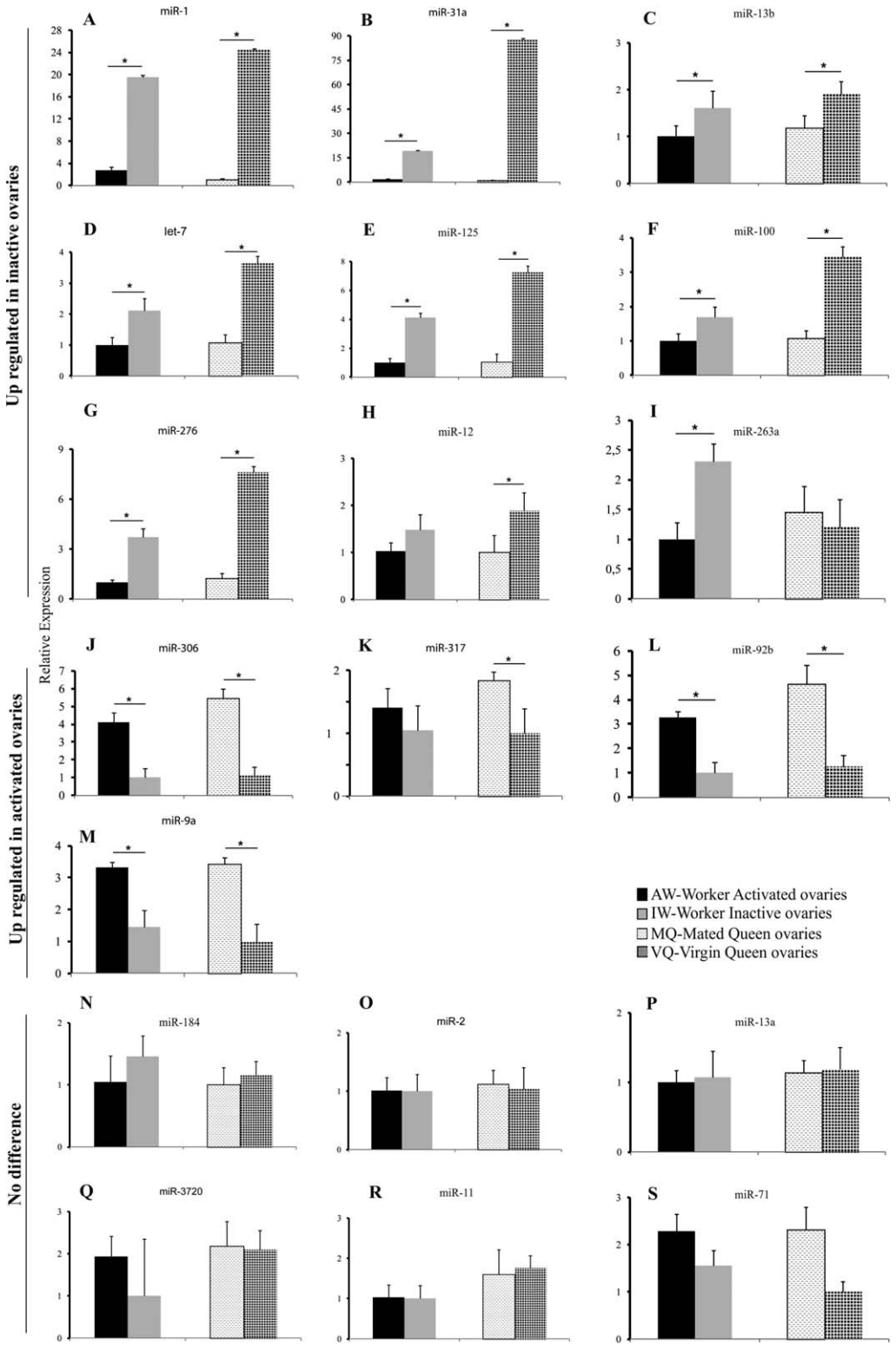


**Figure 2.** Relative expression of microRNAs (miRNAs) up-regulated in inactive ovaries (A–I) and activated ovaries (J–M), as well as miRNAs equally expressed in both types of ovaries (N–S). qPCR was performed using honeybee ovaries under different conditions, including activated workers (AW), inactive workers (IW), mated queens (MQ) and virgin queens (VQ). All tested miRNAs were extracted from the RNA-Seq library constructed using the activated worker ovaries. *U5*, *miR-184* and *miR-2* were used as reference genes in the qPCR assays. The results are shown as the means of three different biological samples (pools) and three technical replicates.

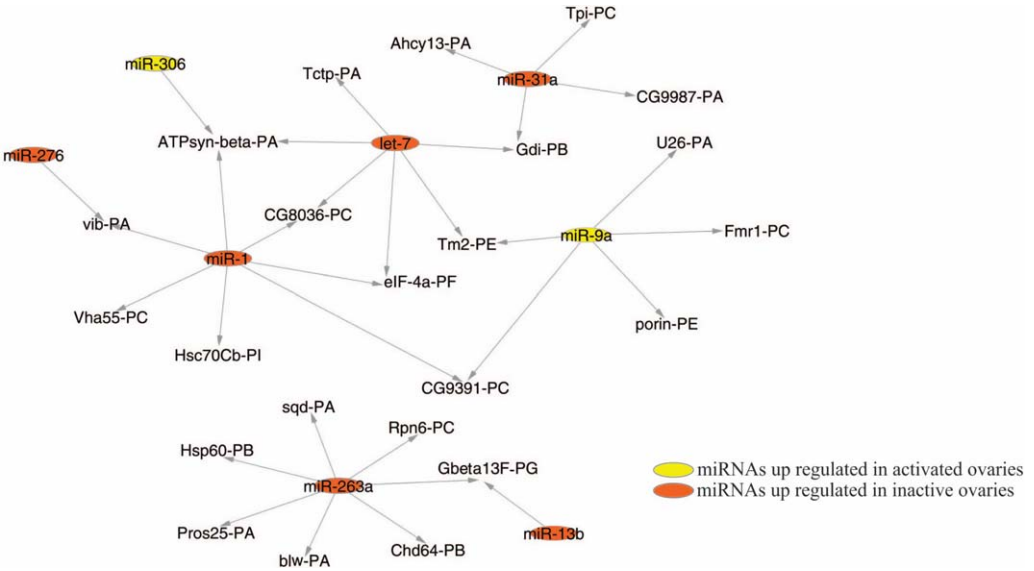


**Figure 3.** Predictive regulatory network of the microRNAs (miRNAs) in the activated and inactive ovaries of the honeybee workers and their predicted targets localized in the differentially expressed protein databank provide by Cardoen *et al.* (2012) (Table S3). Ahcy13-PA, Adenosylhomocysteinase at 13; ATPsyn-beta-PA, ATP synthase-β subunit; blw-PA, bellwether; Chd64-PB, Calponin homology domain-like; eIF-4a-PF, Eukaryotic initiation factor 4a; Gbeta13F-PG, G protein β-subunit 13F; Gdi-PB, GDP dissociation inhibitor; Hsc70Cb-PI, Heat shock protein 70kD, C-terminal domain b; Hsp60-PB, Heat shock protein 60; porin-PE, porin; Pros25-PA, Proteasome α2 subunit; Rpn6-PC, Regulatory particle non-ATPase 6; sqd-PA, squid; Tctp-PA, Translationally controlled tumour protein orthologue; Tm2-PE, Tropomyosin 2; Tpi-PC, Triose phosphate isomerase.

(*miR-276, miR-1, miR-263a, miR-13b, miR-31a* and *let-7*) met all these requisites (perfect seed matches and a free energy $<-20$ kcal/mol). Only *miR-125* and *miR-100,* which were up-regulated in inactive ovaries, did not present predicted targets in the database used (Fig. 3).

The search recovered several genes as miRNA targets that are involved in the activated ovary status of honeybees. This result is congruent with those presented by Cardoen *et al*. (2012). Additionally, we searched for the enriched biological processes based on gene ontology (GO) using DAVID 6.7 (Dennis *et al*., 2003) to better understand the results (Table 1). 'Cytoskeleton organization', 'generation of precursor metabolites and energy', 'ATP synthesis coupled proton transport', 'germ cell development', 'ion transport', 'dorsal/ventral axis specification', 'response to abiotic stimulus' and 'negative regulation of translation' were the main biological processes identified. The most highly expressed miRNA in the deep-sequenced ovary library, *miR-306,* was also highly expressed in all activated ovaries. This miRNA regulates one gene, ATP synthase-β subunit (ATPsyn-beta-PA; XP_624156.3; GB52736-RA), which is involved in 30 biological processes, as noted by our GO search. In addition, this target gene is regulated by two additional miRNAs, *let-7* and *miR-1*, that together regulate 10 targets involved in ovary reproductive status in workers. However, for these 10 predicted targets [Tropomyosin 2 (*Tm2*-PE); Translationally controlled tumour protein orthologue (*Tctp*); CG8036; GDP dissociation inhibitor (*Gdi*-PB); CG939; Heat shock protein 70kD, C-terminal domain b (*Hsc70Cb*-PI); vibrator (*vib*-PA); Regulatory particle non-ATPase 6 (*Rpn6*-PC); Calponin homology domain-like (*Chd64*-PB); Adenosylhomocysteinase at 13 (*Ahcy13*-PA); CG9987; *U26*-PA], related biological processes were not identified.

## Discussion

This study examined the extreme reproductive capacity of queen ovaries and the ability of the worker to reverse its sterility state and attain a reproductive state, to help understand the complex process that governs reproduction in the eusocial insect *A. mellifera*. It should be noted that the factors influencing the reproductive division of labour in honeybees are established early in development by signals from specific diets that are sensed by different genes for determining queens and workers. It is also important to consider that adults are sensitive to environmental signals that, beyond a physiological state, may destroy the delicate reproductive equilibrium of the colony. The number of novel microRNAs and researchers involved in their identification and description continue to increase since the discovery of those noncoding molecules two decades ago. Studies determining their biological function has demonstrated their importance in many biological processes (Kozomara & Griffiths-Jones, 2013). To gain insights into the genetic nonprotein-coding factors driving changes in the

**Table 1.** Gene ontology analysis of the microRNA targets

| Term | No. of genes | *P*-value | Benjamini (corrected *P*-value) | Genes |
|---|---|---|---|---|
| Cytoskeleton organization | 5 | 2.20E-02 | 4.40E-01 | eIF-4a; Fmr1; Gbeta13F; Pros25; **sqd** |
| Generation of precursor metabolites and energy | 4 | 1.40E-02 | 6.00E-01 | ATPsyn-beta; Tpi; Vha55; blw |
| Germ cell development | 4 | 1.90E-02 | 4.20E-01 | eIF-4a; Fmr1; blw; sqd |
| Microtubule cytoskeleton organization | 4 | 3.30E-02 | 4.90E-01 | eIF-4a; Fmr1; sqd; Pros25 |
| Ion transport | 4 | 4.70E-02 | 5.50E-01 | ATPsyn-beta; blw; porin; Vha55 |
| Microtubule-based process | 4 | 7.00E-02 | 6.40E-01 | eIF-4a; Fmr1; sqd; Pros25 |
| Energy coupled proton transport, down electrochemical gradient | 3 | 7.70E-03 | 9.50E-01 | ATPsyn-beta; Vha55; blw |
| ATP synthesis coupled proton transport | 3 | 7.70E-03 | 9.50E-01 | ATPsyn-beta; Vha55; blw |
| Ion transmembrane transport | 3 | 8.30E-03 | 8.00E-01 | ATPsyn-beta; Vha55; blw |
| Proton transport | 3 | 9.70E-03 | 7.20E-01 | ATPsyn-beta; Vha55; blw |
| Hydrogen transport | 3 | 1.00E-02 | 6.30E-01 | ATPsyn-beta; Vha55; blw |
| Dorsal/ventral axis specification | 3 | 1.20E-02 | 6.00E-01 | eIF-4a; Fmr1; sqd |
| ATP biosynthetic process | 3 | 1.50E-02 | 5.60E-01 | ATPsyn-beta; Vha55; blw |
| ATP metabolic process | 3 | 1.50E-02 | 5.30E-01 | ATPsyn-beta; Vha55; blw |
| Purine nucleoside triphosphate biosynthetic process | 3 | 1.60E-02 | 5.00E-01 | ATPsyn-beta; Vha55; blw |
| Purine ribonucleoside triphosphate biosynthetic process | 3 | 1.60E-02 | 5.00E-01 | ATPsyn-beta; Vha55; blw |
| Nucleoside triphosphate biosynthetic process | 3 | 1.60E-02 | 4.80E-01 | ATPsyn-beta; Vha55; blw |
| Ribonucleoside triphosphate biosynthetic process | 3 | 1.60E-02 | 4.80E-01 | ATPsyn-beta; Vha55; blw |
| Purine nucleoside triphosphate metabolic process | 3 | 1.80E-02 | 4.70E-01 | ATPsyn-beta; Vha55; blw |
| Purine ribonucleoside triphosphate metabolic process | 3 | 1.80E-02 | 4.70E-01 | ATPsyn-beta; Vha55; blw |
| Ribonucleoside triphosphate metabolic process | 3 | 1.80E-02 | 4.50E-01 | ATPsyn-beta; Vha55; blw |
| Nucleoside triphosphate metabolic process | 3 | 1.90E-02 | 4.30E-01 | ATPsyn-beta; Vha55; blw |
| Purine ribonucleotide biosynthetic process | 3 | 2.40E-02 | 4.50E-01 | ATPsyn-beta; Vha55; blw |
| Purine ribonucleotide metabolic process | 3 | 2.70E-02 | 4.60E-01 | ATPsyn-beta; Vha55; blw |
| Ribonucleotide biosynthetic process | 3 | 2.70E-02 | 4.60E-01 | ATPsyn-beta; Vha55; blw |
| Transmembrane transport | 3 | 2.90E-02 | 4.70E-01 | ATPsyn-beta; Vha55; blw |
| Ribonucleotide metabolic process | 3 | 2.90E-02 | 4.70E-01 | ATPsyn-beta; Vha55; blw |
| Purine nucleotide biosynthetic process | 3 | 3.50E-02 | 5.00E-01 | ATPsyn-beta; Vha55; blw |
| Purine nucleotide metabolic process | 3 | 3.80E-02 | 5.10E-01 | ATPsyn-beta; Vha55; blw |
| Oxidative phosphorylation | 3 | 3.90E-02 | 5.10E-01 | ATPsyn-beta; Vha55; blw |
| Dorsal/ventral pattern formation | 3 | 4.10E-02 | 5.10E-01 | eIF-4a; Fmr1; sqd |
| Nucleotide biosynthetic process | 3 | 5.00E-02 | 5.50E-01 | ATPsyn-beta; Vha55; blw |
| Nucleobase, nucleoside, nucleotide and Nucleic acid biosynthetic process | 3 | 5.40E-02 | 5.60E-01 | ATPsyn-beta; Vha55; blw |
| Nucleobase, nucleoside and nucleotide biosynthetic process | 3 | 5.40E-02 | 5.60E-01 | ATPsyn-beta; Vha55; blw |
| Monovalent inorganic cation transport | 3 | 6.50E-02 | 6.20E-01 | ATPsyn-beta; Vha55; blw |
| Axis specification | 3 | 8.20E-02 | 6.70E-01 | eIF-4a; Fmr1; sqd |
| Response to abiotic stimulus | 3 | 9.20E-02 | 7.00E-01 | Hsp60; Tpi; porin |
| Negative regulation of translation | 2 | 7.80E-02 | 6.70E-01 | Fmr1; sqd |

*eIF-4a*, Eukaryotic initiation factor 4a; *ATPsyn*-beta, ATP synthase, $\beta$ subunit; *Hsp60*, Heat shock protein 60; *sqd*, squid; *Vha55*, Vacuolar H+-ATPase 55kD subunit; *porin*, porin; *Pros25, Proteasome $\alpha$2 subunit*; *Gbeta13F*, G protein $\beta$-subunit 13F; *tpi*, Triose phosphate isomerase; *blw*, bellwether.

physiological state of ovary activity in eusocial insects, we performed deep sequencing of small RNAs from activated ovaries of honeybee workers as an supporting data to investigate the reproductive profiles of both castes, workers and queens.

Our single RNA-Seq library revealed that more than 50% of the currently known honeybee miRNAs are expressed in activated worker ovaries. A bioinformatics analysis along with in-house-developed tools revealed that half of the detected miRNAs are conserved amongst arthropods and/or metazoans and are highly expressed ($\sim 1.5 \times 10^6$ reads), and the other half are species-specific and are expressed at lower levels ($\sim 1.5 \times 10^4$ reads). In general, evolutionarily ancient miRNAs are widely and abundantly expressed in a variety of tissues and play key roles throughout development (Lyu *et al*., 2014), whereas nonconserved miRNAs tend to be expressed at lower levels and are associated with spatiotemporally specialized biological processes (Axtell, 2008). This phenomenon also occurs in honeybees. We observed that various miRNAs exhibiting different levels of conservation are consistently expressed in the ovaries. These miRNAs probably maintain the reproductive state of the ovaries or act as potential controllers of the overall physiology. For example, *miR-184* and *miR-2* were highly and equally expressed regardless of the reproductive condition of the organ.

The functions of some of the miRNAs are well known from the current literature. However, for others, the function can only be deduced from computational target prediction using the standard method, as previously suggested (Bleazard *et al*., 2015). *miR-184-3p, miR-71-5p, miR-2-3p* and *miR-11* were amongst the top 15 highly expressed miRNAs in the active ovaries

of the workers (Fig. 1), but these miRNAs appear to be equally important in both castes because they were similarly abundant in all of the ovaries tested (qPCR data comparisons, Fig. 2). However, *miR-306-5p, miR-1-3p* and *miR-276-3p* were also amongst the top 15 (Fig. 1) but were differentially expressed depending on the reproductive condition of the ovary (Fig. 2), suggesting a specific function in this process. Therefore, we suggest that the analysed miRNAs are markers of ovary activation independent of the caste.

Amongst the miRNAs that were up-regulated in the activated ovaries and that have known functions, the most highly expressed miRNA in the library is *miR-306. miR-306* targets *Bag of marbles*, a gene involved in germ cell differentiation. In *D. melanogaster* this miRNA controls stem cell differentiation during the spermatogenesis process (Eun *et al*., 2013; Gancz & Gilboa, 2013). Additionally, we have shown that this library includes miRNAs directly related to ovary reproductive physiology, germ cell production and follicle development. For example, *miR-1* is involved in the deposition of the transcription factor Zelda in the developing oocyte (Fu *et al*., 2014). Some miRNAs are directly related to cell division, such as *miR-12*, which targets MCT1 (monocarboxylate transporter) in *Aedes aegypti* (Osei-Amo *et al*., 2012), and *miR-31*, which suppresses the expression of CDK2 (cyclin-dependent kinase 2), regulates the cell cycle and is essential for meiotic arrest in mammals (Valastyan *et al*., 2015).

The highly conserved *miR-184* exhibited stable expression in all tested groups, AW, IW, VQ and MQ (Fig. 2N), and is widespread in metazoans. Several studies have demonstrated that *miR-184* is ubiquitously expressed in a myriad of animal tissues throughout development and adulthood (Wienholds & Plasterk, 2005; Chen & Rajewsky, 2007; Zhang *et al*., 2012; Nunes *et al*., 2013). In particular, the pleiotropic roles of *miR-184* in the regulation of multiple steps in oogenesis and early embryogenesis are well known in *D. melanogaster*. *miR-184* is a maternal effect gene that plays key roles in embryogenesis, the determination of the anteroposterior axis, embryo cellularization and stem cell determination (Iovino *et al*., 2009).

In *D. melanogaster*, the cluster of *miR-2* forms the largest miRNA family, which contains eight members (Marco *et al*., 2012). In *A. mellifera*, the *miR-2* cluster is composed of *miR-2-1, miR-2-2, miR-2-3, miR-2b, miR-13a, miR-13b* and *miR-71.* A previous study reported that members of the *miR-2* family, along with *miR-11,* share similarities in seed sequences, targeting several developmental coding genes during morphogenesis, organ development, cell differentiation and cell fate commitment (Marco *et al*., 2012). Curiously, *miR-71-5p, miR-2-3p* and *miR-11* each presented similar levels of expression in the ovaries under the different reproductive statuses in both castes, whereas *miR-13b* expression was significantly increased in the inactive ovaries of the queens and workers compared with activated ovaries. Lozano *et al*. (2015) observed that the members of the *miR-2* cluster target different genes despite their high levels of similarity. One member of the *miR-2* cluster, *miR-13b*, was described as targeting Histamine receptor H1 (*hr-h1*), a gene that functions in the transition of nurse to forager in *A. mellifera*. Another member of this cluster, *miR-71* (together with *miR-239*), participates in specific steps of the insulin/insulin-like signalling pathway, regulating the ageing process (Smith-Vikos & Slack, 2012). Taken together, we conclude that members of this cluster indirectly control the ovary activation process by modifying the behaviour and development in adult workers given that these miRNAs appear to participate in the ageing and senescence processes.

The marked morphological differences between the ovaries of an egg-laying queen and the ovaries of a sterile worker are reduced during worker ovary activation, at least when individual ovarioles are considered. In activated worker ovaries, Ronai *et al*. (2016) observed well-developed oocytes. However, they also found cells suffering from developmental apoptosis in the nurse chamber. This fact might explain the presence in our library of *miR-31a* and *miR-263,* which have been described as related to the apoptotic process by Stark *et al*. (2003) and Hilgers et al. (2010), respectively. In the qPCR experiments, we obtained consistent results for both miRNAs in queens, but in workers miR-263 clearly exhibited increased expression in the activated ovaries. According to Ronai *et al*. (2016), an observation of intense cell death in the ovaries of workers exposed to the queen pheromone indicates a nonreproductive state.

Regardless of the expression level, any single miRNA may have multiple gene targets. The particular function of a miRNA in a given tissue depends on the set of target genes that is co-expressed. This scenario is even more intriguing in honeybees because the genomic template of the queens and workers is the same, with the differences emerging from (epi)genetic regulatory levels and the consequent differential expression profiles (Lyko *et al*., 2010; Foret *et al*., 2012).

The qPCR experiments demonstrated that miRNA expression in the ovaries depends on the reproductive status of these organs but not on the caste. Thus, to explore the regulation by the miRNAs involved in ovary activation in honeybee workers, we constructed an illustrative network based on our data generated from miRNA expression (qPCR) and data from Cardoen *et al*. (2012) (Fig. 3). The predictive regulatory network consistently correlated the two types of data. The most highly

expressed miRNA, *miR-306,* targets ATPsyn-beta-PA, an essential molecule for many biological processes (Table 1). This protein, which is present in the database published by Cardoen *et al*. (2012), could explain the expression levels of *miR-306* observed in our data base. Within hours, *miR-306* senses the absence of the queen in the colony (results not shown). Although additional experiments are necessary, this result is valuable given that it allows data from different sources to be coherently processed and compared. Cardoen *et al*. (2012) used a proteomic approach to conclude that gene expression is markedly different between workers with active and inactive ovaries. In our study, we compared castes and reproductive conditions by analysing differentially expressed miRNAs from a library generated from active worker ovaries.

The construction of a miRNA library from the active ovaries of honeybee workers, the expression analysis and the prediction of targets provided a portrait of the sensitive reproductive equilibrium that maintains both castes in apparent harmony in a colony, where all members assume their roles in this sophisticated society at the right time. This is the first time that specific miRNAs have been tested with the intention of characterizing the reproductive state of the ovaries in sterile and egg-laying workers, as well as virgin and mated queens, based on the miRNAs expressed in active worker ovaries.

In the studied case, environmental stimuli drive the expression of miRNAs that subsequently regulate the reproductive status of worker bee ovaries by mimicking the ovary of a fertile queen. Therefore, we conclude that the miRNAs are deeply involved in this process and that the reproductive state is independent of caste, as suggested by the expression of these regulatory molecules.

## Experimental procedures

### *Honeybee biological samples*

All of the samples were obtained from Africanized honeybee colonies at the Experimental Apiary of the Universidade de São Paulo in Ribeirão Preto, Brazil. A total of four different ovary samples was used in this study: (1) a pool of AW ovaries; (2) a pool of IW ovaries IW; (3) individual MQ ovaries and (4) individual VQ ovaries (Table 2). To sample the IW ovaries, the frames with emerging broods from the queenright colonies were placed in an incubator (34 °C and 80% relative humidity). Newly emerged workers were paint marked and returned to their original colony. After 4 days, the marked workers were collected and their ovaries dissected in cold saline (NaCl 0.9%). To induce ovary activation in the workers and therefore sample AW ovaries, the colonies were dequeened, and all queen-rearing cells were destroyed. Frames with an emerging brood were placed in an incubator (34 °C and 80% relative humidity), and the newly emerged workers were paint marked and returned to their original orphaned colony. After 10 days, the marked workers were collected and their ovaries dissected in cold saline (NaCl 0.9%). We considered activated ovaries as those containing developed oocytes; otherwise, ovaries were classified as inactive. VQ ovaries were obtained from newly emerged queens. MQ ovaries were obtained from the queens removed from the above-mentioned dequeened colony. The sampled bees were anaesthetized by incubation in a refrigerator for 10 min. All of the dissections and tissue samplings were performed on ice. For total RNA isolation, we used 500 μl TRIzol® (Invitrogen, Carlsbad, CA, USA), following the protocol suggested by the manufacturer. RNA samples were quantified using a NanoDrop spectrophotometer (NanoDrop Tech. Inc., Wilmington, DE, USA).

A small RNA-Seq library was generated from a pool composed of six AW. The total amount of RNA in the AW sample was approximately 8 μg. Single-end sequencing was performed by DNA Vision (Charleroi, Belgium, http://www.dnavision.com) on an Illumina Genome Analyzer with Solexa technology (Illumina Inc. San Diego, CA, USA).

For gene expression validation, quantitative real-time PCR (qRT-PCR) was performed using the first-strand complementary DNA (cDNA) prepared from the ovary pools: three pools each of AW, IW, MQ and VQ.

### *Computational analysis of the sequenced small RNA library*

Base calling from the sequenced small RNA library was performed at the bioinformatic facilities at DNA Vision. The computational processing of the reads consisted of the following steps: (1) initial sequence quality filtering allowing no more than 50% unidentified nucleotides; (2) rRNA read filtering based on matches against the SILVA database (Quast *et al*., 2013) using BOWTIE2 (Langmead & Salzberg, 2012); (3) read trimming based on a Phred-like (base-calling peak predictions) quality score >25; and (4) sequence adaptor clipping using CUTADAPT (Martin, 2011) and SCYTHE v. 0.981 (https://github.com/vsbuffalo/scythe). After each of these preprocessing steps, alignments against the

**Table 2.** Description of the samples collected for the experiments

| Sample abbreviation | Features | Number of ovaries | Small RNA-Seq library | qRT-PCR |
|---|---|---|---|---|
| IW | Inactive workers' ovaries – day 4 of adulthood | 15 pairs | – | Three samples |
| AW | Activated workers' ovaries – day 10 of adulthood | Six pairs | One sample | Three samples |
| VQ | Virgin queen – just emerged | One pair | – | Three samples |
| MQ | Mated queen | One pair | – | Three samples |

qRT-PCR, quantitative real-time PCR.

*A. mellifera* genome (assembly version 4.5) were performed using the reads that were not previously aligned at each previous alignment step. These genomic alignments were performed using TopHat (Trapnell *et al*., 2009). Finally, the split alignments were excluded. All of the remaining alignment results were concatenated and transformed into a proper format to be used by miRDeep2 (Friedlander *et al*., 2012), which provided the counts of reads mapped to each known *A. mellifera* miRNA (considering miRBase v. 19) as their correspondent digital expression. We considered miRNAs with $>10$ mapped reads to be expressed (Table S1). The library can be found at http://www.ncbi.nlm.nih.gov/sra under the accession number SRR1613229.

### *cDNA synthesis and qRT-PCR analyses*

A total of 2 μg of each RNA sample (AW, IW, MQ and VQ) was used for reverse transcription using NCodeTMmiRNA First-Strand CDNA (Invitrogen, Carlsbad, CA, USA). We used a PCR kit (Invitrogen) for synthesis and qRT-PCR following the manufacturer's protocol. From the RNA-Seq data, we selected 19 miRNAs for validation using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The selected miRNAs were amongst the most expressed in the library, with more than 500 reads (Table S1) and associated with oogenesis or belonging to a cluster with at least one highly expressed member. miRNAs from the same cluster might have different roles and expression patterns. The reactions were performed in a final volume of 20 μl containing 10 μl SYBR® Green Master Mix 2x (Applied Biosystems), 2 μl cDNA, 7.2 μl water and 0.4 μl (10 pmol/μl) of each primer (specific forward primer and the Universal qPCR reverse primer provided in the NCode kit). In addition to the three biological replicates, each sample was also analysed in triplicate (technical replicates). The efficiency of each primer pair used in qPCR was assessed by constructing a standard curve using 1:10 serial dilutions of cDNA. The PCR conditions were 2 min at 50 °C; 10 min at 95 °C; and 40 cycles of 15 s at 95 °C and 33 s at 60 °C. The relative expression was calculated using the comparative Threshold cycle ($C_T$) method according to the previously reported mathematical model (Livak & Schmittgen, 2001). In the present study, the stable expression observed for *U5* spliceosomal RNA, *miRNA-184* and *miRNA-2* was confirmed by Bestkeeper analysis (Tichopad *et al*., 2004), which classified these as suitable reference genes for qPCR reactions using honeybee ovary tissues. The high but very stable expression of *miR-184* in *D. melanogaster* ovaries also suggested its use as a normalizer in qPCR, as described by Ge *et al*. (2015). Thus, the average $C_T$ was used to normalize cDNA levels. The quantitative data were analysed using a *t*-test. All of the pairwise comparisons were considered significant when $P < 0.05$. The primer sequences used in the qPCR experiments are shown in Table S2.

### *Prediction of miRNA targets and functional annotation based on GO analysis*

To investigate the potential interactions between the validated miRNAs and the gene targets, we compared our data with a previously published set of ovary proteomic data (Cardoen *et al*., 2012). These authors identified 224 protein spots as differentially expressed between activated and inactive ovaries from *A. mellifera* worker bees. We analysed those data, excluding the redundancies, and the number of proteins was reduced to 165 unique proteins. Using the protein accession numbers, we identified the respective nucleotide information and recovered the predicted or validated 3′UTR sequences of the 153 mRNAs from the RefSeq-GenBank database (National Center for Biotechnology Information). Mature sequences of the studied miRNAs showing the differential expression patterns in the worker ovaries (AW and IW) were recovered from miRBase, release 19. Both the 3′UTR and the miRNA sequences were used as inputs to run the target predictions using the RNAHybrid tool (Kruger & Rehmsmeier, 2006). To this end, the same criteria used by Nunes *et al*. (2013) were applied, ie we accepted only the duplexes of miRNA : mRNA presenting perfect seed matches (Bartel, 2009). We restricted our results to interactions presenting a free energy $< -20$ kcal/mol.

The predicted honeybee and fruit fly target gene orthologues were identified by reciprocal best hits using BLAST tools (Altschul *et al*., 1990). To explore the functional role played by the predicted target genes, we searched for enriched biological processes as described in the GO database using David 6.7 (Dennis *et al*., 2003) with the default parameters. A predictive regulatory network was generated using Cytoscape v. 2.7.0 (Shannon *et al*., 2003; Table S3).

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Table S1.** Number of reads mapped in mature microRNAs of *Apis mellifera* in activated ovaries of worker honeybees. Cut-off $\geq$10 mapped reads.

**Table S2.** Primer sequences used in quantitative real-time PCR.

**Table S3.** Proteins differentially expressed in activated and inactive ovaries of honeybee workers found by Cardoen *et al*. (2012). It was eliminated the retudance, and was found the corrent XP and XM, according to the RefSeq-GenBank database (National Center for Biotechnology Information) in September 2014.