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Original article

Circadian clock genes are differentially modulated during the daily cycles and chronological age in the social honeybee (*Apis mellifera*)

Fabiano C. P. Abreu[1], Flávia C. P. Freitas[1], Zilá L. P. Simões[2]

[1]Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, Brazil
[2]Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, Brazil

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Abstract – The circadian clock is an advantageous adaptive system that enables organisms to predict and anticipate the daily environmental changes. The circadian rhythms are generated molecularly through the expression of clock genes, based on autoregulatory feedback loops. Honeybees are an excellent model to investigate how the circadian rhythms are modulated accordingly to the social context, behavioral plasticity, and task-related activities. Here, we show how the clock genes behave during the daily cycles in adult worker heads of *Apis mellifera*. Our results point to the clock genes *period* and *cryptochrome* as essential regulators of the circadian rhythms associated to the behavioral maturation in this social insect. We also identified putative miRNA-target and protein-protein interactions involving honeybee clock genes, indicating regulatory networks behind the adjustment of the molecular clock.

circadian clock / clock genes / circadian rhythms / honeybees / miRNAs

1. INTRODUCTION

Temporal coordination of diverse physiological and behavioral processes allows organisms to adapt to environmental daily cycles. Internal clocks behind the temporal coordination process orchestrate the expression of specific genes and generate circadian rhythms, resulting in cyclic biological activities around the 24-h period (Dunlap et al. 2004; Bell-Pedersen et al. 2005). The molecular clockwork is highly conserved throughout evolution, and the ongoing identification of their molecular components reveals remarkable similarities and differences between vertebrates, invertebrates, and plants (Reppert and Weaver 2000; Young and Kay 2001; Vansteensel et al. 2008).

Circadian rhythms in insects are well known and a wide range of studies demonstrated their intimate relationship with physiological processes as eclosion, ovoposition, ecdysis, metamorphosis, locomotor activity, behavior, and mating (reviewed by Saunders et al. 2002). Comparative analysis within insects from different orders revealed differences in the neuroanatomy of the circadian clock, genetic components, and modes of regulation of the circadian rhythms (Sandrelli et al. 2008; Tomioka and Matsumoto 2010). Interestingly, the molecular clock of honeybees and ants is more similar to mammals than to *Drosophila melanogaster* (Rubin et al. 2006; Weinstock et al. 2006; Ingram et al. 2012; Sadd et al. 2015). The honeybee *cryptochrome* (*cry-m*), for

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Corresponding author: F. Abreu,
fabiano.genetica@usp.br
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example, is an ortholog to its correspondent in mammals and is absent in *Drosophila* genome (Rubin et al. 2006).

The molecular studies of the clockwork genes on *D. melanogaster* and *Mus musculus* (reviewed by Panda et al. 2002) and their expression pattern (Rubin et al. 2006) were the references for the construction of the theoretical knowledge on clockwork genes of *Apis mellifera*. It consists in autoregulatory feedback loops caused through the oscillatory expression of canonical clock genes in groups of neurons in the central nervous system (Bloch et al. 2003; Rubin et al. 2006; Fuchikawa et al. 2017). Molecular and phylogenetic analyses identified eight clock genes in *Apis mellifera*: *period* (*per*), *cryptochrome* (*cry-m or mammalian-type cry*), *clock* (*clk*), *cycle* (*cyc*), *timeout 2* (*tim2*), *par domain protein 1* (*pdp1*), *vrille* (*vri*), and *clockwork orange* (*cwo*) (Weinstock et al. 2006; Rubin et al. 2006; Rodriguez-Zas et al. 2012). The autoregulatory cycle starts with the formation of the heterodimer CLK/CYC that binds to regulatory sequences and activates the expression of *per* and *cry-m*. The products of *per* and *cry-m* accumulate at night in the cytoplasm of oscillatory neurons and act as negative elements forming the heterodimer PER/CRY-m that enters the nucleus and prevents their own expression binding to CLK/CYC heterodimer (Rubin et al. 2006). The functional domains of VRI and PDP1 are highly conserved to *D. melanogaster* domains. In honeybees, *clk* and *cyc* present putative binding sites for both VRI an PDP1, suggesting another autoregulatory feedback loop (Cyran et al. 2003; Rubin et al. 2006; Bloch 2010). The role of *cwo* and *tim2* in the modulation of the circadian rhythms in Hymenoptera is still unknown. Beyond the canonical clock genes, hundreds of transcripts exhibit circadian oscillations around the zeitgeber in brains of nurses and foragers of honeybees reflecting the clock-controlled genes (Rodriguez-Zas et al. 2012).

The post-transcriptional regulation performed by microRNAs also plays an important role in modulating the oscillatory expression of clock genes (Kojima et al. 2011, Luhur et al. 2013). MicroRNAs were found to be rhythmic in *Drosophila* brain, mouse CNS, mouse retina, and *Arabidopsis*, and some of them are directly involved in modulating the circadian period and response to light (Cheng et al. 2007, Xu et al. 2007, Jung et al. 2007, Yang et al. 2008, Sire et al. 2009, Kadener et al. 2009). In honeybees, a set of microRNAs were identified as involved in caste determination (Weaver et al. 2007; Chen et al. 2010; Ashby et al. 2016), memory and learning (Cristino et al. 2014; Qin et al. 2014), foraging (Behura and Whitfield 2010; Liu et al. 2012a, b), embryogenesis (Pires et al. 2016; Freitas et al. 2017), and reproductive status (Macedo et al. 2016). However, there are no studies showing whether miRNAs affect the circadian system in honeybees.

Due to highly organized society, extraordinary cognitive capacity, and complex behavior, honeybees are an excellent model to investigate how the circadian rhythms can be influenced by the social interactions (Meshi and Bloch 2007; Bloch 2010; Shemesh et al. 2010; Eban-Rothschild et al. 2012; Nagari and Bloch 2012). The social synchronization is as important as light and temperature to entrain the clock of honeybees, and adult workers can exhibit different activity rhythms depending on the colony ambient and contact to conspecifics (Frisch and Koeniger 1994; Nagari and Bloch 2012; Fuchikawa et al. 2016). Further, the behavioral plasticity of honeybees associated to the division of labor is thought to be closely related to modulation of circadian rhythms (Bloch et al. 2001; Shemesh et al. 2007; Shemesh et al. 2010). Here, we tested if the daily oscillation of canonical clock genes changes in workers with different ages by assessing the expression profiles of the genes *per*, *cry-m*, *clk*, *cyc*, *cwo*, *tim2*, *vri*, and *pdp1* in heads of young (3- and 7-day-old) and old (15- and 25-day-old) bees. We used computational analysis to predict microRNAs as regulators of clock genes and to rebuild a protein interaction network that illustrates the physical interactions between the products of the canonical clock genes and other proteins. Our aim was to show how the circadian rhythm plasticity is related to the

complex behavior of honeybees through clock gene expression in a special colony, using an Africanized *A. mellifera* population from a tropical region. Studies focused on the molecular clock of social insects certainly will shed light on how the eusociality could be related to the evolution of internal clocks.

2. MATERIAL AND METHODS

2.1. Bee samples

We worked with experimental colonies in the "single-cohort colony" model, which allow us the control of the adulthood, behavioral maturation, and sample bees of the same age. We removed honeycomb frames containing pupae, close to eclosion, from source colonies of Africanized honeybees (*Apis mellifera*) from the experimental apiary of the Department of Genetics at the Faculdade de Medicina de Ribeirão Preto (Ribeirao Preto, Brazil) and transferred them to dark incubator (32 ± 0.5 °C, 55 ± 5% relative humidity) to obtain emerging bees with 0–24 h. Approximately 1500 newly emerged worker bees were paint-marked on the thorax and introduced in an experimental observation hive provided with a virgin queen and a frame with pollen, honey, brood, and empty cells where the queen could lay eggs. The observation hive was kept in a dark room (to prevent light stimulation) with constant temperature (28 °C ± 1). An exit was built in the experimental hive so that the bees could fly away from the hive, collect food (forage), and experience the sunlight. Worker bees with 3, 7, 15, and 25 days old were collected at four zeitgeber times (7, 12, 17, 22 h) under red light. Sampled bees ($n = 3$–5 per zeitgeber time) were individually immersed in liquid nitrogen and had their entire heads collected for subsequent extraction of total RNA. The sampling procedure was performed from September to October, during the spring. We kept the colony in the absence of light as soon as we started the experiment and did not use an artificial light-dark (LD) regime to synchronize the colony population. However, the colony was susceptible to be socially synchronized by free-flying foragers that experienced the sunlight outside, so as by the nurse bees during the experimental flies.

2.2. RNA extraction for mRNA expression profile

Total RNA was extracted from entire heads of individually sampled bees using Trizol reagent (Invitrogen) accordingly to the manufacturer protocol. The extracted RNA was treated with RNAse-free DNAse (DNase I Amplification Grade—Invitrogen), and the total RNA was quantified using the NanoDrop® ND-1000 Spectrophotometer V3.03 (NanoDrop Technologies).

For mRNA expression analysis, an aliquot of 1.5 μg of total RNA was reverse transcribed using an oligoDT and the specific enzyme SuperScript™ II Reverse Transcriptase (200 U/μL, Invitrogen), as described by the manufacturer. Specific oligonucleotides were designed for *per*, *cry-m*, *clk*, *cyc*, *tim2*, *cwo*, *pdp1*, and *vri* using Primer3 tool (http://bioinfo.ut.ee/primer3-0.4.0/) and validated through qPCR standard curve method (Livak and Schmittgen 2001) to evaluate their efficiency. Oligonucleotide sequences are listed in Supplemental Table S1. RT-cDNA samples were used as templates for qPCR amplification of the clock genes with the SYBR® Green Master Mix 2× (Applied Biosystems) and 7500 Real-time PCR System (Applied Biosystems). The gene expression was normalized against the *ribosomal protein L32* (*rpl32*), as suggested by Lourenço et al. (2008) and calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Statistical analyses were performed using the one-way ANOVA test and Tukey's pairwise comparison ($p < 0.05$).

2.3. Gene regulatory network for miRNA-mRNA interactions

We selected a region of 1000 pb downstream to the stop codon of clock genes to represent the 3′ UTR and performed the search for miRNA binding sites using the RNAhybrid tool (Kruger and Rehmsmeier 2006). We selected this parameter because the sequences of untranslated regions (5′ and 3′ UTRs) are not available for every gene in

the current version of honeybee genome (The Honeybee Genome Sequencing Consortium 2006; Elsik et al. 2014). To reduce false-positive miRNA-target interactions, we performed the search for miRNA binding sites in 3′ UTRs of *Drosophila* genes and considered for further analysis the miRNA-target interactions conserved in both species. This approach has proven to be efficient in detecting functional miRNA-binding sites in honeybees as demonstrated by publications of our group (Cristino et al. 2014; Freitas et al. 2017). We considered only the interactions that are based on the seed region of miRNAs (2-7nt), free energy ≤ -20 Kcal/mol, and p value < 0.05.

2.4. Protein-protein interaction network

To build a putative protein-protein interaction (PPi) network, we retrieved proteins that interact with clock genes in *Drosophila* and *Mus musculus* from BioGrid repository (version 3.4.138) (Chatr-aryamontri et al. 2017). The PPis involving conserved genes in honeybee were used to build a PPi network using Cytoscape tool, version 3.4.0 (Shannon et al. 2003). Functional analysis of PPi network was carried out based on information available at GeneOntology Consortium (www.geneontology.org), PANTHER Classification System (www.phanterdb.org) ($p \leq 0.05$), and FlyBase (www.flybase.org).

3. RESULTS

3.1. Chronological and daily patterns of clock gene expression during the adult development of *A. mellifera*

Our quantitative PCR (qPCR) analysis revealed that the clock genes *per*, *cry-m*, *clk*, *cyc*, *tim2*, *pdp1*, *vri*, and *cwo* are expressed in heads of adult workers in the chronological ages of *A. mellifera* (Figure 1). The clock genes *per*, *cry-m*, and *cwo* exhibited a similar expression pattern showing higher transcriptional levels in heads of old bees (25-day-old) compared to younger bees. Conversely, the clock gene *pdp1* showed an opposite expression pattern, with higher transcriptional levels in the heads of young bees (3-day-old) compared to older bees. The average transcriptional levels of *clk* were higher in heads of 25-day-old bees compared to 3- and 15-day-old bees, but showed no significant differences related to 7-day-old bees. For *cyc* and *vri*, 15-day-old bees showed differences in their average transcriptional levels compared to 3-day-old and 7-day-old bees, respectively. The average transcriptional levels of *tim2* did not vary with different ages.

Daily expression patterns of clock genes in heads are evident in at least one chronological age. Based on the amplitude of the expression profile variation, we classified the transcriptional oscillations as absent, present, or attenuated present (Figures 2 and 3). Transcriptional oscillations of *per* and *cry-m* within a day were absent or attenuated present in young bees (3- and 7-day-old) and showed high amplitude in older bees (15- and 25-day-old). Both *per* and *cry-m* presented peaks of expression at the beginning of the day (7 h) and in the middle of the dark phase (22 h) in the heads of 15-day-old bees. In 25-day-old bees, we observed a peak of expression of *per* and *cry2* in the dark phase (22 h). For *clk*, we found a clear transcriptional oscillation in heads of 25-day-old bees with one peak at the light phase (12 h) and another at the dark phase (22h) (Figure 2 (b)). For *cyc*, the transcriptional oscillation was more attenuated in the young bees and similar to *clk* oscillation in 25-day-old bees.

Interestingly, *pdp1* showed a strong daily oscillation in heads of young bees (3-day-old) compared to *vri*, while the opposite was observed for *vri* in old bees (25-day-old) (Figure 3 (a)). Both *pdp*1 and *vri* showed peaks of expression in the light phase of the day (12 h). For *cwo*, we observed transcriptional oscillations in heads of 3-, 15-, and 25-day-old bees (Figure 3 (c)). A peak of *cwo* expression was observed at the light phase (17 h) in heads of 3-day-old and 15-day-old bees, what implies a circadian oscillation at these ages. In 25-day-old bees, the levels of *cwo* are high in the light phase (17 h) and reach a peak of expression at the dark phase (22 h). Transcriptional oscillation of *tim2* was absent within a day

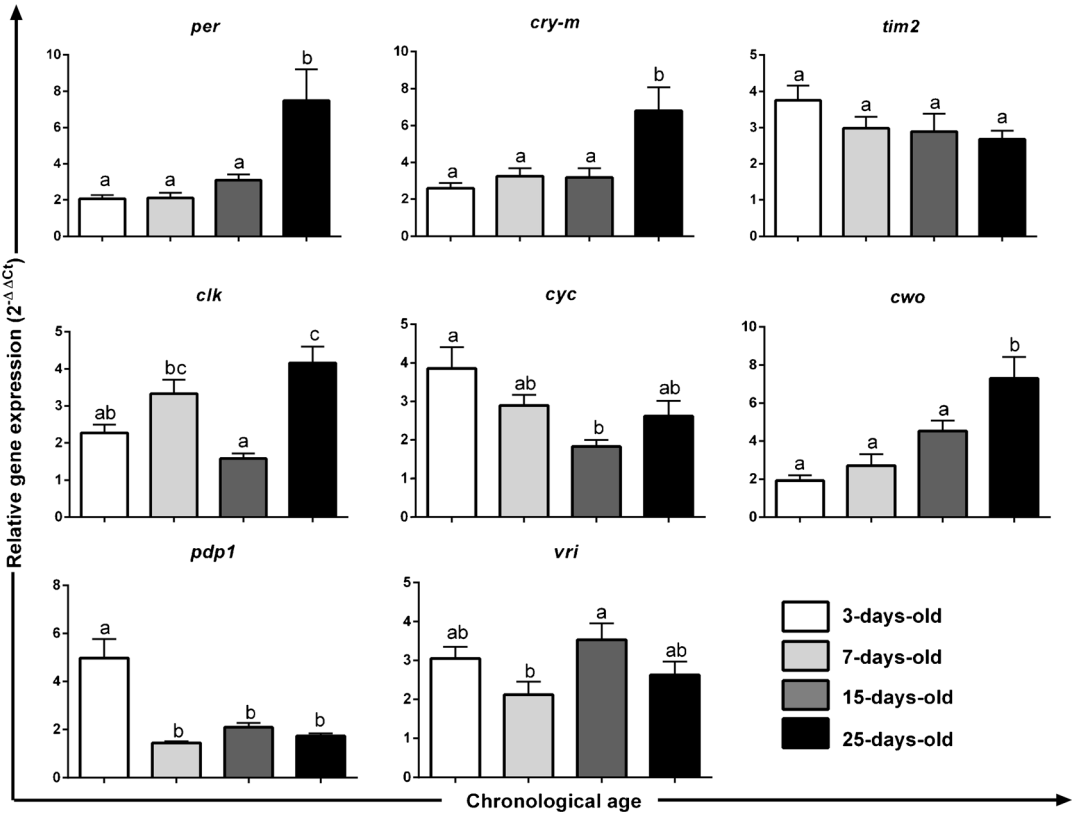

Figure 1. Transcriptional levels of clock genes during the chronological age in worker heads of *A. mellifera*. qPCR was used to obtain the relative gene expression which was normalized to the endogenous gene *rpl32* through the $2^{-\Delta\Delta Ct}$ method. For each adult age, we used the mean expression of clock genes in heads of three to five individuals calculated from each time point collected during the day. Statistical analysis was performed using one-way ANOVA, followed by Tukey's pairwise comparison, $p < 0.05$.

during the chronological age of worker bees (Figure 3 (b)).

3.2. Interaction networks

Our computational analysis revealed that the clock genes *per*, *cry-m*, *clk*, *cyc*, *tim2*, *pdp1*, *vri*, and *cwo* exhibited putative binding sites in their 3′ UTR that are supposed to interact with miRNAs in *A. mellifera.* Due to the high number of miRNA-mRNA interactions recovered by our analysis and taking into account that computational predictions for binding sites of miRNAs can generate false-positive results, we selected miRNAs previously listed in other insects and with a possible role in regulating circadian and neuronal functions at least in *D. melanogaster.* We reconstructed a miRNA-mRNA network using interactions predicted in both species (Figure 4a). The results point to potential miRNAs to regulate the clock genes that are well studied in fruit flies or reported to be involved in honeybee developmental processes, like *let-7*, *miR-8*, *miR-9a*, *miR-34*, *miR-124*, and *bantam.*

We found physical interactions for the clock genes *per*, *cry-m*, *clk*, *cyc*, *tim2*, *pdp1*, and *vri* based on curated data for orthologs in *D. melanogaster* and *M. musculus.* Based in our protein-protein interaction network (Figure 4b), we searched for gene ontology ($p \leq 0.05$) (Supplemental Figure S1) and found biological processes linked to cellular, metabolic, and

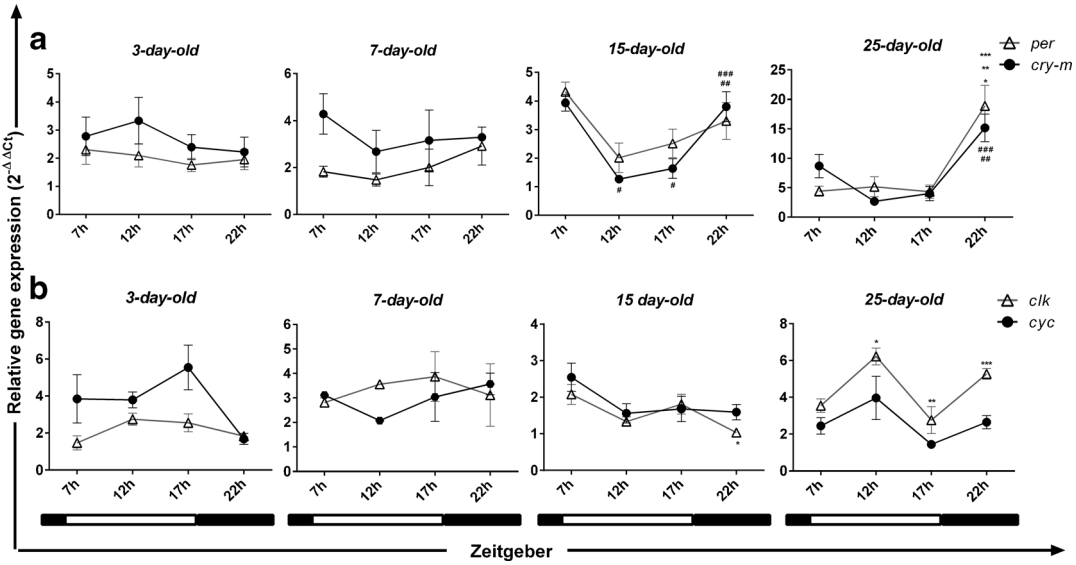

Figure 2. Daily expression patterns of clock genes *per* and *cry-m* (*a*) and *clk* and *cyc* (*b*), involved in a first autoregulatory feedback loop, during the chronological age in worker heads of *A. mellifera*. qPCR was used to obtain the relative gene expression which was normalized to the endogenous gene *rpl32* through the $2^{-\Delta\Delta Ct}$ method. Each zeitgeber time represents the mean expression in heads of three to five individuals. Statistical analysis was performed using one-way ANOVA, followed by Tukey's pairwise comparison, $p < 0.05$. * or # different from 7 h, ** or ## different from 12 h, *** or ### different from 17 h. Black and white bars at the bottom reflect the sunlight hours with dark and light daily phases, respectively.

developmental regulation, mainly related to enzymatic, catalytic, and structural activities.

4. DISCUSSION

In this work, we analyzed the chronological and daily patterns of clock gene expression in heads of *A. mellifera* using four different adult ages representing young (3- and 7-day-old) and old (15- and 25-day-old) bees living in a single-cohort colony. The characteristics of a single-cohort colony as same-aged bees made possible to test the expression of clock genes are influenced by the chronological age and/or behavior. Our analysis showed that the average daily expression of *per* is higher in heads of old bees (25-day-old) compared to young bees (3- and 7-day-old) (Figure 1), consistent with Toma et al. (2000). In addition, our analysis pointed to higher daily expression of *per* in heads of 25-day-old bees compared to 15-day-old bees. We observed these same features for *cry-m* and *cwo*, which have not been demonstrated before. The higher transcriptional levels of *per*, *cry-m*, and *cwo* in heads of 25-day-old bees may be related to the intense forager activity that depends on strong circadian rhythmicity. A previous study did not observe differences in the expression level of *per* in brains of young bees (8- to10-day-old) compared to old bees (23- to 25-day-old) using single-cohort colonies (Bloch et al. 2001). Although it seems contradictory, other findings support that expression levels of *per* transcripts in the central nervous system of worker bees are not strictly linked to their chronological age but depend on other factors such as precocious foraging, a characteristic of both single-cohort colonies reared in field or laboratory (Toma et al. 2000; Bloch et al. 2001). In our analysis, average daily expression of *per*, *cry-m*, and *cwo* in heads of young bees (3–7-day-old) compared to potential foragers (15-day-old) showed no difference (Figure 1). The absence of foragers in the initial population of our single-cohort colony (composed of newly emerged workers) likely affects *per*, *cry-m*, and *cwo* mRNA levels, and the expression of these genes

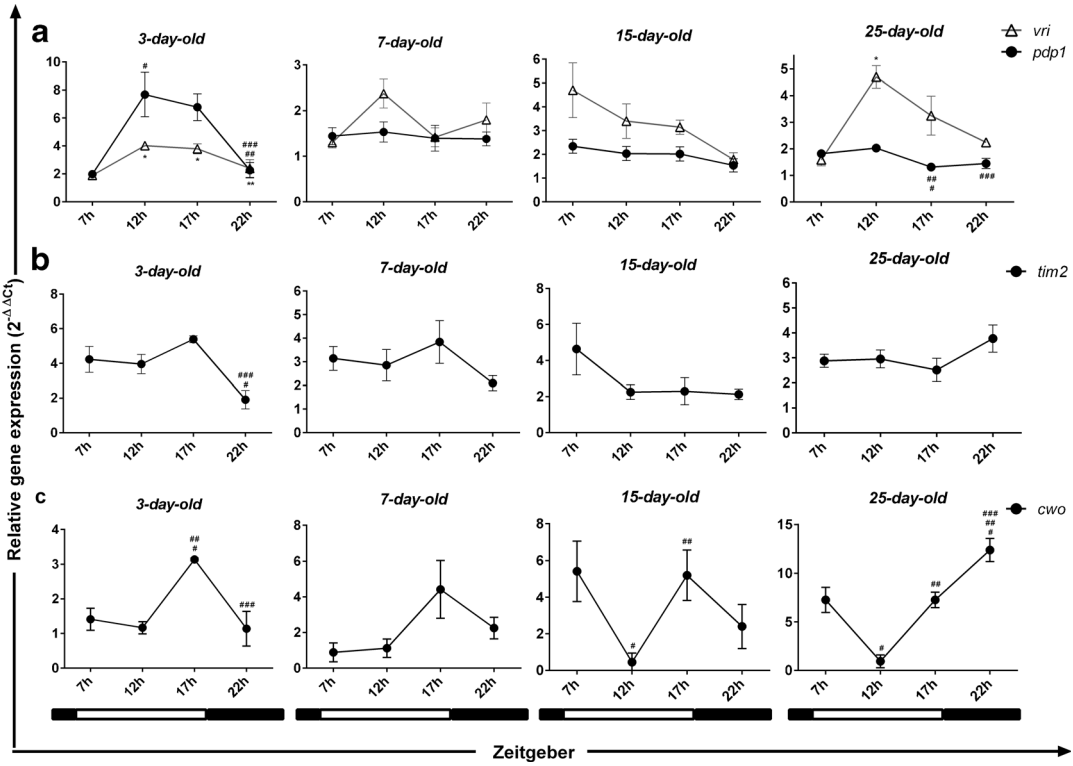

Figure 3. Daily expression patterns of clock genes *pdp1* and *vri* (*a*), *tim2* (*b*), and *cwo* (*c*) during the chronological age in worker heads of *A. mellifera*. qPCR was used to obtain the relative gene expression which was normalized to the endogenous gene *rpl32* through the $2^{-\Delta\Delta Ct}$ method. Each zeitgeber time represents the mean expression in heads of three to five individuals. Statistical analysis was performed using one-way ANOVA, followed by Tukey's pairwise comparison, $p < 0.05$. * or $^{\#}$ different from 7 h, ** or $^{\#\#}$ different from 12 h, *** or $^{\#\#\#}$ different from 17 h. Black and white bars at the bottom reflect the sunlight hours with dark and light daily phases, respectively.

reaches higher levels earlier in adult life as a signal to prepare young bees to forage. We did not find variation in the average levels of *per*, *cry-m*, and *cwo* comparing 15-day-old bees and 3–7-day-old bees; nonetheless, we observed a striking difference between their daily transcriptional fluctuation in heads at least for *per* and *cry-m* (Figure 2 (a)). An absence or attenuated oscillation in daily expression of both genes found in young bees compared to the strong oscillation in old bees is consistent with other studies (Bloch et al. 2001; Shemesh et al. 2007). Our results suggest that the expression levels of *per* and *cry-m* are associated with the behavioral plasticity in honeybees and that the oscillations in their expression reflect the division of labor between young and old bees. While young bees (nurses) work arrhythmically around the clock inside the colony in brood-care activities, old bees (foragers) need to be strongly rhythmic to develop complex tasks as foraging, sun navigation, learning, memory, and dance language. In contrast, Fuchikawa et al. (2017) found that the oscillation on protein levels of *per* is similar in brains of behaviorally rhythmic foragers and arrhythmic nurses. This observation in soma with our analysis suggests that cycling on proteins levels does not require cycling of the transcripts in arrhythmic nurses. Taken together, our analysis supports the idea that the ontogeny of circadian rhythms is closely linked to the division of labor in social insects and transcriptional levels of *per* and *cry-m*. Our results point to the clock genes *per* and *cry-m* as candidate molecular markers of behavioral maturation in honeybees.

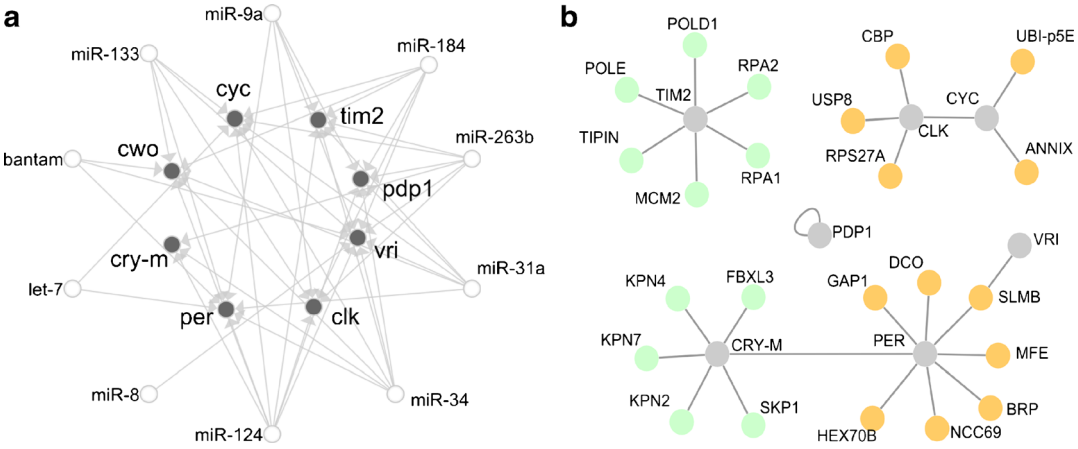

Figure 4. a miRNA-mRNA interaction network. The network illustrates the putative miRNAs that were predicted to bind to 3′ UTRs in transcripts of clock genes in the genome of *A. mellifera*. The prediction was based on previously determined parameters ($\Delta G \leq -20$ Kcal/mol; p value < 0.05). White nodes and gray nodes represent the predicted miRNAs and the target clock genes, respectively. **b** Protein-protein interaction network representing the putative physical interactions between clock genes and other proteins in *A. mellifera.* The analysis was based on curated data available for *D. melanogaster* and *M. musculus* in the BioGRID online tool (http://thebiogrid.org/). Physical interactions based on orthology with *D. melanogaster* correspond to orange nodes linked to clock genes *per*, *cyc*, *clk*, and *vri* (gray nodes). Interactions based on orthology with *M. musculus* represent the green nodes linked to clock genes *cry-m* and *tim2* (gray nodes).

Noteworthy, expression levels of *clk* do not oscillate within a day in young bees (3- or 7-day-old) but it does oscillate in old bees (25-day-old). Our data contrasts to a previous study that reported absence of oscillation in *clk* in forager bees (Rubin et al. 2006). The daily transcriptional levels of *cyc* are synchronized with *clk* in 25-day-old bees and it is unlikely that the moderate oscillations observed are due to the circadian influence. In mammals, *tim2* is expressed in retinoid cells, which indicates that this gene is related to photoreceptor function (Sangoram et al. 1998; Takumi et al. 1999). In another Hymenoptera, *Ceratosolen solmsi*, *tim2* has an arrhythmical expression in males that live inside the syconium of flowers in the absence of light. In young females that leave the syconium earlier, *tim2* showed a rhythmic expression around the day, suggesting that the oscillation of this gene is light dependent (Gu et al. 2014). However, our data show that *tim2* did not fluctuate during all the chronological age of worker bees (Figure 3 (b)). This result is in accordance with those reported by Rubin et al. (2006). Further experiments are necessary to address how *tim2* participates in the circadian mechanism in honeybees.

Here, we showed for the first time the expression profiles of *vri* and *pdp1* in heads of honeybees. Besides the classical role of *pdp1* and *vri* in the modulation of circadian rhythms, non-circadian roles were found for *pdp1* and *vri* in embryos and larval development of flies (George and Terracol 1997; Reddy et al. 2000; Szuplewski et al. 2003). The strong transcriptional fluctuations detected for *pdp1* during the beginning of adult life and later in 25-day-old bees for *vri* point to different roles during the development of adult workers (Figure 3 (a)). The high transcriptional levels of *pdp1* in young bees (3-day-old) may be related to their nutritional status, once it was reported that this gene is affected by the availability of food during larval development in fruit flies (Reddy et al. 2006). As reported in the literature, the diet and feeding regime regulate the internal clock of organisms and we suggest that the amount and differential uptake of nutrients during the chronological age of honeybees affect directly *pdp1* expression (Chaudhari et al. 2017).

Analysis pointed to the conservation of protein domains between Hymenoptera *cwo* and its ortholog in *D. melanogaster*, indicating that this gene has conserved the same function within insects (Kadener et al. 2007; Lim et al. 2007; Matsumoto et al. 2007; Ingram et al. 2012; Rodriguez-Zas et al. 2012). We observed that the high transcriptional levels of *cwo* at 17 h in heads of old bees (Figure 3 (c)) are similar to those observed by Rodriguez-Zas et al. (2012). Similar fluctuation patterns of *cwo* expression profile from 7 to 17 h were observed in heads of 15-day-old and 25-day-old bees, but not at 22 h. We cannot discard that in a social environment, not all the bees will present the same circadian phase, at least when gene expression is considered. In a social community, the members behave differently due to the opportunity to interact each other. The same is supposed to occur with the circadian rhythm. An example is the expression of *cwo* that demonstrates sensitivity to colony social organization and varies when individual tests were performed. This enables us to say that a single-cohort colony affected the transcriptional modulation since it has a peculiar demographic distribution, at least at the initial experimental phase.

The identification of miRNAs in circadian neurons of *D. melanogaster* suggested that these small non-coding RNAs might regulate the stability or the translation of *clk*, *vri*, and *cwo* (Kadener et al. 2009). Additionally, computational simulations have demonstrated that miRNAs are involved in the modulation of the circadian rhythms altering its amplitude and periodicity (Nandi et al. 2009). Kadener et al. (2009) also described several miRNAs upregulated in circadian neurons of *D. melanogaster* when the enzyme Dicer was disrupted by the knockdown. Some conserved miRNAs found in our analysis are linked to neuronal functions: *let-7*, *miR-8*, *miR-9a*, *miR-34*, *miR-124*, and *bantam* (reviewed by Asgari 2013). Overexpression of *bantam*, a potential regulator of *clk*, in adult neurons of fruit flies leads to longer rhythmic periods (Kadener et al. 2009). Overexpression or deletion of *let-7* affects the length of circadian rhythms (Chen et al. 2014). It is suggested that *let-7* forms a regulatory cycle in the circadian clock being activated indirectly by CLK/CYC through the ecdysteroid pathway and negatively regulates the expression of *cwo*. Further, *let-7*, *miR-8*, and *miR-34* are essential players in preventing neurodegeneration process and aging in *D. melanogaster* (Liu et al. 2012a, b; Chawla et al. 2016). *miR-92a* has an oscillatory expression in circadian neurons of fruit flies and modulates the neuronal excitability through regulation of *sirt2* (Chen and Rosbash 2016). The miRNAs *miR-31a*, *miR-124*, *miR-133*, *miR-184*, and *miR-263b* (Figure 4a) found in our analysis have been detected in the central nervous system of *D. melanogaster* (Kadener et al. 2009), which opens the opportunity to investigate experimentally how these miRNAs are involved in the modulation of circadian rhythms in honeybees.

Beyond the post-transcriptional regulation of clock genes, physical interactions at translational level are important to regulate the stability of clock proteins in the nucleus or in the cytoplasm (Weber et al. 2011; Reischl and Kramer 2011; Crane and Young 2014). Moreover, the canonical clock proteins not only act as transcriptional factors in the circadian machinery but also interact with other products to regulate non-circadian functions. Our protein-protein interaction network (Figure 4b) revealed physical interactions between clock proteins and non-clock proteins related to the biological processes: post-translational regulation, phosphorylation, ubiquitination, and degradation. The *discs overgrown* (DCO), also called *doubletime* (DBT), is one of the proteins found to phosphorylate PER contributing to modulate its translational levels in the cytoplasm (Price et al. 1998; Kloss et al. 1998; Cyran et al. 2005; Reischl and Kramer 2011; Weber et al. 2011). The protein *supernumerary limb* (SLMB) recognizes the phosphorylated site in PER and acts in recruiting other proteins to direct PER to degradation through the ubiquitin-proteasome system (Ko et al. 2002; Chiu et al. 2008). The proteins USP8, RPS27A, and UBI-p5E involved in the ubiquitin-proteasome system interact with CLK and CYC suggesting that a conserved degradation machinery is essential to the clearance of clock gene products. We also observed a physical interaction between VRI and SLMB, suggesting a possible non-circadian role during embryogenesis, as reported in the literature and in a specialized

database (FlyBase) (George and Terracol 1997; Szuplewski et al. 2010). Another possible role linked to the generation of circadian rhythms was observed for PER and BRP (*bruchpilot*) interaction; it is known from the literature that BRP is associated with the synaptic circadian plasticity in the visual system of fruit flies (Woźnicka et al. 2015). The *brp* gene is also described to be involved in functions related to adult locomotor behavior and short-term memory (Wagh et al. 2006; Knapek et al. 2011).

The physical interactions recovered for TIM2 and CRY-m, orthologous in mammals, revealed proteins related to metabolism and DNA replication processes. We reinforce here that all proteins found to interact physically with TIM2 and CRY-m based on *M. musculus* data present an ortholog in the honeybee, suggesting similar interactions. We observed that all proteins found to interact with TIM2 have an essential role in regulating the chromosome integrity and DNA replication (Gotter et al. 2000; Benna et al. 2010). CRY-m showed two physical interactions with FBXL3 and SKP1, probably originating a complex to polyubiquitinate CRY-m and direct it to degradation. This result suggested a possible role of this complex to regulate CRY-m stability and modulate the circadian rhythms (Xing et al. 2013; Yoo et al. 2013). The physical interactions for CRY-m also revealed proteins involved in the nuclear import, the Karyopherins KPN2, KPN4, and KPN7, as candidates to participate in CRY-m translocation from the cytoplasm to nucleus. Taken together, the reconstruction of protein-protein interaction network allowed us to extend our view on the possible interactions at a translational level that potentially regulate the stability of clock proteins and even establish non-circadian functions along the development of honeybees.

Finally, our work gave us the opportunity to discuss how the circadian clock genes are modulated in the central nervous system of Africanized honeybees reared under special conditions, taking into account that they are localized in a different geographic region compared to the honeybees living in the temperate climate. Here, we added new elements to understand how the conserved circadian machinery, analyzed under these circumstances, so as affected by climatic, geographic, nutritional, and social contexts, modulates the circadian rhythms and the behavioral plasticity in social insects, especially honeybees.

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AUTHORS' CONTRIBUTION

All authors have contributed equally to the work: Fabiano C.P. Abreu and Zilá L.P. Simões elaborated the idea of this work, experimental procedures were performed by Fabiano C. P. Abreu, and Flavia C.P. Freitas performed the computational analyses.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest The authors declare that they have no conflict of interest.

Les gènes de l'horloge circadienne sont modulés de façon différentielle au cours des cycles quotidiens et de l'âge chronologique chez l'abeille domestique (*Apis mellifera*)

horloge circadienne / gene horloge / rythme circadien / abeille / miRNAs

Die Expression von Genen der circadianen Uhr ist differentiell moduliert in Bezug auf die Tageszyklen und das chronologische Alter der sozialen Honigbiene (*Apis mellifera*)

circadiane Uhr/ Uhr-Gene / circadiane Rhythmen / Honigbiene / micro RNAs

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