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Chronobiological plasticity in Honeybee and its association with differences in the organization of the molecular pathway between central and peripheral clocks: the brain and flight muscle tissues

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DEDICATION

وَأَوْحَىٰ رَبُّكَ إِلَى النَّحْلِ أَنِ اتَّخِذِي مِنَ الْجِبَالِ بُيُوتًا وَمِنَ الشَّجَرِ وَمِمَّا يَعْرِشُونَ (Verse 16:68)

And thy Lord taught the bee to build its cells in hills, on trees, and in "men's" habitations;

ثُمَّ كُلِي مِنْ كُلِّ الثَّمَرَاتِ فَاسْلُكِي سُبُلَ رَبِّكِ ذُلُلًا ۚ يَخْرُجُ مِنْ
بُطُونِهَا شَرَابٌ مُخْتَلِفٌ أَلْوَانُهُ فِيهِ شِفَاءٌ لِلنَّاسِ ۗ إِنَّ فِي ذَٰلِكَ لَآيَةً لِقَوْمٍ يَتَفَكَّرُونَ (Verse 16:69)

Then to eat of all the produce (of the earth), and follow the ways of thy Lord made smooth: there issues from within their bodies a drink of varying colors, wherein is healing for men: verily in this is a Sign for those who give thought.

Sura النحل - An-Nahl
(Hive Bee found in Holy Quran)

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Together with her, I like to express my gratitude to my principal PhD supervisor, Prof. Carmelo Rapisarda (from the Department of Agri-food and Environmental Systems Management of the University of Catania, Italy), without whom I would not have been able to conclude this PhD. Since the beginning, he trusted my project proposal and was always ready to help me whatever the problem was. I am grateful for his supernatural help, unlimited availability, great advice and generosity.

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ABSTRACT

Circadian rhythms govern the behavior, physiology, and metabolism of living organisms, enabling them to anticipate changes of environmental conditions. Within Insects, in honey bee's colonies an ontogeny in circadian rhythms of behavior was suggested to be related to an age- based division of labor, to best serve their social organization: young adult tend to perform tasks in the nest, nursing brood around the clock, with no circadian rhythms for the 2–3 first weeks, and then shift to foraging outside for the remainder period of life, enabling visiting flowers at time of maximal nectar and pollen availability. In addition to light, social cues is one of the primary "zeitgeber" that entrain the locomotor activity rhythm in honeybees, as notably showed by the robustness of circadian activity of young nurses soon after their release far from social interactions with the conspecifics. Such context dependent plasticity in circadian behavior was often associated with a variation in the main core clock genes mRNA expression profile in the brain, but it has not been yet examined whether such variations extend to other body tissues of the honeybee. In this thesis, I identified for the first time oscillating transcription of core clock genes in the flight muscle of workers, which temporal cycling pattern differed significantly in LD and under DD, in a task rather than age dependent manner. Although the autonomy of such oscillator from central orchestration has yet to be determined, the consistent temporal cycling pattern of mRNA level enriched for genes highly involved in muscle metabolism, suggest exciting future opportunities to discover additional clock–controlled genes in the honeybee. The ensemble of progresses, might give answer to the kind of relationships between different clocks, involving eventually total and partial autonomy, especially in such an excellent model in chronobiology: ***the Honeybee***.

Key words: Honeybee, flight muscle, temporal cycling pattern, clock genes, metabolic genes, division of labor.

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1. INTRODUCTION

Almost all organisms are exposed to the earth's rotation around its axis and around the sun with predictable changes in the geophysical environment. More precisely, the periodic variation in the daily light and dark span and the seasonal changes in day length give rise to numerous biological rhythms in physiology and behavior. These rhythms are observed in the whole organism but also in single organs and recur at approximately regular intervals: from 20 to 28 h (=circadian); < 24 h (=ultradian); > 24 h (=infradian) and ± 1 year (=circannual) (reviewed by Halberg, 1969). They are synchronized in a meaningful way, allowing organisms to efficiently anticipate periodic events and to place themselves adaptively in the appropriate spatio-temporal conditions of the planet, by adjusting organism physiology and behavior to such changes and thereby optimize survival.

The study of biological rhythms, known as chronobiology, has been focused mainly on biological rhythms that are synchronized to the environmental 24 h-cycle, no doubt for their predominance in nature and their relevant control of a variety of biological processes. In fact, it is common to see: honey bee visiting flowers precisely at the same time of the day, even when nectar source is removed; plants that continuously open and close their leaves regularly, also if placed under constant darkness; fruit flies eclosing in the morning, regardless of constant laboratory conditions, and exhibiting same rhythm pattern in mating or egg laying activity. Similar relevant circadian coordination is conserved across a wide range of *taxa* including prokaryotes, birds, mammals or fishes. The most obvious explanations given for these conservation is that circadian rhythms are ubiquitous, they evolved under the influences of periodic geophysical environmental forces and have been fine-tuned under strong selective pressure.

In order to determine whether these rhythms are directly driven by a periodic environmental stimulation (exogenous rhythm) or whether they are an intrinsic characteristic of a circadian system of the organism (endogenous rhythm), applied and basic research questions have been used to uncover both the mechanisms and impact of circadian rhythms in tandem. Results suggest that most of the daily fluctuations in behavioral and physiological variables are not merely passive responses to the daily alternation of light and dark, as they persist even when no external time cues are present, such as in constant darkness. Indeed, circadian rhythms are driven by an endogenous clock (or a pacemaker) and have an endogenous period of approximately 24 h. In the

absence of a synchronizing “Zeitgeber” (time giver) (Aschoff, 1981), these rhythms “free run” with an intrinsic natural period, which shortens or lengthens closely to 24 hours and exhibit a “temperature compensation”, that allows their period length to remain over a wide range of physiological temperatures. Such fundamental characteristics account largely in providing organisms with a “Temporal organization” or “Time keeping” or “Biological clock”, allowing them to perform biological functions “at the right time of the day” by coordinating a functional relationship between various metabolic processes within the organism and the periodic factors that belong to world outside (reviewed by Sanders *et al.*, 2002).

1.1 Basic principles of circadian rhythm

In the modern era, the endogenous nature of circadian timekeeping has been illustrated through the use of several experimental models, most of them developed on pheno - type based screen of mutagen-exposed flies (Konopka & Benzer, 1971). Such studies allowed the discovery of 3 period mutants: long (*perL*, ± 29 h), short (*perS*, ± 19 h) and arrhythmic (*per0*), which all mapped to a single locus, called *period (per)*, on the X-chromosome, that could affect the persistence of either eclosion rhythm or locomotor activity of flies. Such findings facilitated further researches (Bargiello *et al.*, 1984; Reddy *et al.*, 1984) that confirm in *Drosophila* the same *period* as the first clock gene, indeed for the large protein of more than 1200 amino acids that it encodes. Later, such studies and others applying transgenic experiments in rodents, has characterized several other clock genes and reinforced basic understandings of the molecular mechanisms that coordinate the rhythmic regulation of circadian behavioral and physiological phenotypes (as reviewed by Hardin., 2005).

At this stage, endogenous circadian clocks became defined as internal molecular timekeeping mechanisms that reside in diverse range of cell types in a variety of organisms, from unicellular bacteria to vertebrates, and regulate the timing of behavior and physiology (Dunlap *et al.*, 2004). Their key features are the following.

1.1.1 Entrainment

The most distinguishing characteristic of a circadian rhythm is the stability of the period.
In the presence of the 24 h environmental cycle, where the light-dark periods follow the cyclic pattern of the solar day, organisms adjust their circadian pattern to this dominant environmental entrainer or zeitgeber (Pittendrigh & Minis, 1964). The most important and universal entraining agent is the light-dark cycle generated by the earth's rotation. Numerous studies have investigated the effect of photic entrainment upon organisms. It seems that under experimental conditions, the animal when housed within a 24 h light-dark (LD) cycle, with 12 h of light and 12 h of darkness (LD: 12:12), the entrained clock tend to follow such zeitgeber time in a very stable way, allowing a behavior period length of 24 h too (Aschoff, 1960; Mrosovsky *et al.*, 1992). Other findings revealed that increasing the light intensity under constant light (LL) lengthens the circadian period of nocturnal animals and shortens that of diurnal ones (Aschoff, 1979a). This supports the idea that, in contrast to others factors, light intensity has the largest effect on the free-running period (Aschoff, 1979a, 1979b). In addition, light induced phase shifts also depend on the properties of the light pulse (duration and irradiance) (Daan & Pittendrigh, 1976).
The most important and universal entraining agent is the light-dark cycle generated by the earth's rotation; non-photic cues exists too, such as temperature cycle, food availability, social interactions, which, although playing a secondary role, are able to provide stimuli and entrain the clock to the driving oscillation of the surrounding environment of the organism.
So, through entrainment, the zeitgebers do not create rhythms but do determine their placement in time.

1.1.2 Free-running

However, after elimination of external stimuli, the cycles persist with a period of close to, but not exactly 24 h. This persistence seems to indicate the existence of some kind of internal timekeeping mechanism or biological clock (Pittendrigh & Calderola, 1973).

Under such constant conditions of continuous light (LL) or darkness (DD), the period to which the internal biological clock can be entrained tends to be longer or shorter than 24 h, ranging between

20 to 28 h, depending on various factors: intrinsic factors, such as the physiological status of the individual animal (age, hormone levels), and its genetic background (diurnal or nocturnal species), and on external factors (light intensity, temperature) and the physiological state of the organism (feeding conditions, previous experiment etc.) (Aschoff, 1979a, 1979b; Pittendrigh & Daan, 1976). Thus, under such conditions, the "time" is often measured relatively to the endogenous circadian rhythm. Such time, is defined by researchers as circadian time (CT) and corresponds to a relative point during the organism's behavior rhythm (Pittendrigh, 1966).

Interestingly, once a shift in external cues occurs due to phase shifts of the light - dark cycle or travel across time zones for example, what follows are several unstable transitional cycles between the stable periods established under synchronizing LD condition and those of the new environment until rhythms are aligned to the new cues. This re-alignment is called entrainment (Enright, 1981). Generally, the resulting plot of such phases changes, against the circadian time of such perturbations, is considered to give a phase response curve (PRC) for the oscillation (Saunders *et al.*, 1994).

1.1.3 Temperature compensation

Another fundamental property of the circadian clocks is their ability to maintain a roughly 24-hour periodicity over a range of physiological temperatures; this means that they exhibit a sort of "temperature compensation". Such vital characteristic was revealed as universal, occurring across all *taxa*, and of high functionality, as it allows organisms to keep track of time. In fact, most kinds of biological processes in cells are affected by temperature changes, and a clock that speed up and slow down would not be useful. So Thus, through temperature compensation, a circadian clock is able to efficiently resist and to adapt to environmental changes.

Although the understanding and definitions of circadian system have developed as discoveries have been made, a system could not be called "circadian clock" from a chronobiological point of view, if it does not have the three fundamental and linked components as following (Fig. 1):

- **An input pathway**: represents all the corresponding ways of perception of the information coming from the environment, which are necessary for clock synchronization.

- **A central clock or pacemaker**: that generates cell-autonomous and self-sustaining rhythms. It consists of a series of interconnected positive and negative transcriptional and translational feedback loops governing protein stability and degradation in an evolutionary conserved way across organisms (Pittendrigh, 1960).
- **An output pathway**: output pathways that influence physiological function and behavior through gene expression [generally called clo*ck c*ontrolled genes (*ccgs*)], neural and humoral channels.

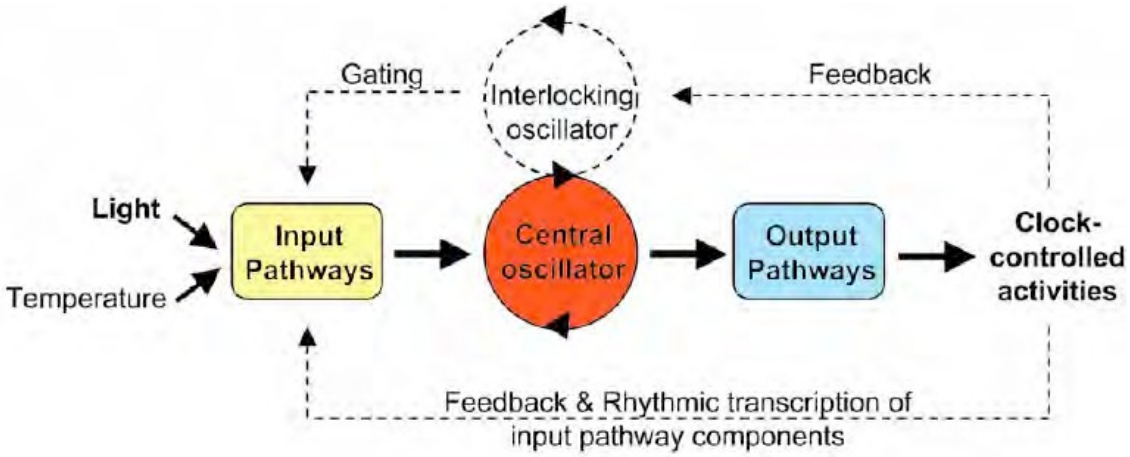


Fig. 1. Fundamental components of the circadian system
"The circadian system consists of three basic elements: an input pathway (yellow), an endogenous pacemaker generating circadian rhythms (red), and an output pathway (blue). Environmental signals, called Zeitgeber (German for "time giver) are transduced to the master clock via input pathways. The input signals are received via receptors and then sent to the central pacemaker that generates oscillations. The output pathway finally translates the oscillation into rhythms such as genome transcription in cyanobacteria or sleep-wake cycles in animals. Some organisms contain more complex circadian clocks (shown as dotted line) that include multiple, interlocking oscillators and positive or negative feedback from clock controlled activities to the pacemaker and/or input components" (Jud, 2009) (figure modified from Gardner *et al.*, 2006).

1.2 Organization of the circadian system in insects

In respect of the fact that *Drosophila melanogaster* Meigen is one of the most studied insects and due to the fact that its clock investigation has led for many years the way to a complexity of molecular aspects of circadian research, that greatly outnumber basic foundations in other insects, the identification and description of the circadian system organization will be mainly focused on these flies, pointing out eventual differences with other insects (especially bees), when judged of relevance and needed to be outlined.

1.2.1 Localization of the master clock (Fig. 2)

In flies, circadian rhythms are regulated by a cell autonomous pacemaker, located bilaterally in the lateral protocerebrum just at the border to the optic lobes and traditionally divided into two main groups of neurons, the lateral neurons (LNs) and the dorsal neurons (DNs) (reviewed by Taghert & Shafer, 2006; Nitabach & Taghert, 2008). Such cell clusters have been determined through cytological staining for the clock gene products and their products, which are prevalently *period* (*per*) and *timeless* (*tim*) (Liu *et al.*, 1992; Saez & Young, 1996). Although both neurons are important, the major influence on the daily locomotor activity and pupal eclosion rhythms confer to the LNs the role of key pacemaker neurons (Zerr *et al.*, 1990; Ewer *et al.*, 1992; Frisch *et al.*, 1994; Helfrich-Forster, 1998). In adult flies, lateral neurons are traditionally divided into a group of four large – ventrolateral neurons (l-LNvs), five small – ventrolateral neurons (s-LNvs), which project directly to the medulla and dorsal protocerebrum, respectively (Stanewsky *et al.*, 1997; Kaneko & Hall, 2000). Both groups have the ability to express the *pigment dispersing factor* (*pd*f) gene, usually used to reveal their projection pattern (Helfrich-Forster, 1995) and well-known as encoding the neuropeptide PDF that plays a key output signal from the circadian clock at peripheral tissues. Unlikely, *pdf* gene is not expressed by the third more dorsally located group, namely the dorsolateral neurons (LNds) (Kaneko & Hall, 2000), which also projects to the dorsal brain. On the other hand, the dorsal neurons (DNs) also split into three groups, which are categorized according to their localization within the dorsal brain, mainly dorsal neurons 1s (DN1s), dorsal neurons 2s (DN2s) and dorsal neurons 3s (DN3s). Kaneko & Hall (2000) and Helfrich-Forster (2002) illustrated the neuronal functioning of DN1 and DN3 projections towards sLNv cell bodies in interconnecting clocks gene expression between neurons.

Although these different groups of clock gene expressing neurons contribute to the control of behavioral activity, it appears through mutant analyses that different aspects of locomotor activity rhythms are not equally regulated by all neurons (Grima *et al.*, 2004), which make some of them being master and others subordinate clocks for behavioral rhythmicity. Under LD cycle, locomotor activity seems to be under the control of two separate oscillators that generate consequently two activity peaks. The 4 s-LNvs appear to be responsible for the morning activity peak (M peak) whereas the LNds and possibly a subset of DN1s regulate the evening peak together (E peak) (Helfrich-Forster, 2000).

These clock neurons also regulate the anticipatory increase in activity corresponding to the M and E peaks. In constant conditions s-LNvs appear to be sufficient to provoke robust activity rhythms, indicating a more important role for these clocks in DD (Grima *et al.*, 2004). However, the LNds and the 5th s-LNv are thought to be capable for modulating the phase of the activity pattern. In flies, projections sent from sLNvs into the dorsal brain, which contains the rhythmically released neuropeptide PDF (Park *et al.*, 2000), drives the locomotor activity rhythm (Renn *et al.*, 1999; Helfrich-Forster, 1995) in a circadian manner and synchronize the timing of different clocks.

This central oscillator receive light input from retinal photoreceptors in the compound eyes and extra-retinal photoreceptors within the brain, allowing synchronization with the environmental light-dark cycles, and possess multiple output pathways to control diverse endocrine, autonomic and behavioral functions, particularly locomotor activity (Fig. 2).

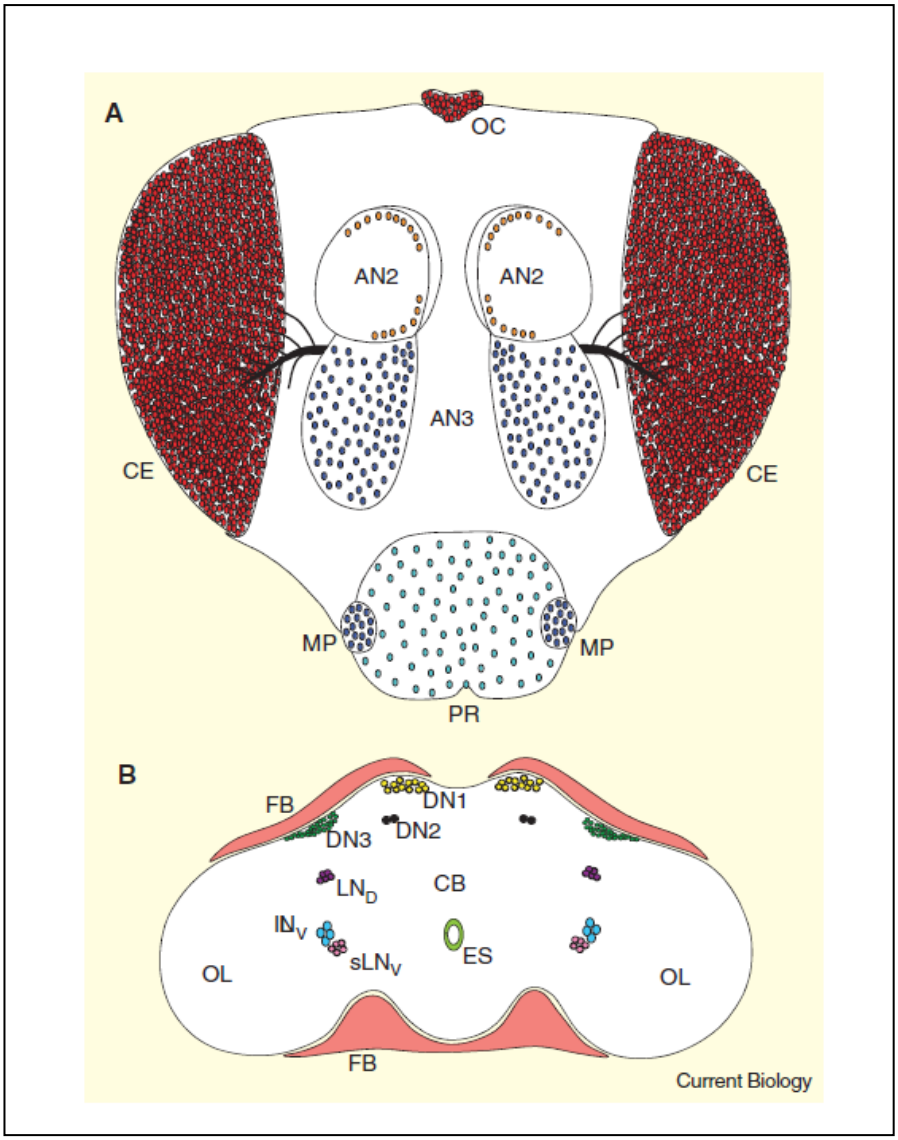


Fig. 2 - Circadian oscillators in *Drosophila* head (A)
External structures containing circadian oscillators. A frontal view of a *Drosophila* head is shown. OC, ocelli; CE, compound eyes; AN2, second antennal segment; AN3, third antennal segment; MP, maxillary palps; PR, proboscis. (B) Oscillator cells within and surrounding the brain. A frontal section through a *Drosophila* brain and surrounding tissues is shown. CB, central brain; OL, optic lobes; FB, fat body; ES, esophagus; LND, dorsal lateral neurons; lLNv, large ventral lateral neurons; sLNv, small ventral lateral neurons; DN1, dorsal neuron 1s; DN2, dorsal neuron 2s; DN3, dorsal neuron 3s (Illustrated by Hardin, 2005).

1.2.2 The core molecular clock

Since the first clock gene *period* has been cloned in *Drosophila*, thousands of studies have been published identifying several other clock genes that play an important role in the timekeeping mechanism (reviewed by Hardin, 2005). As a consequence, several molecular models have been

assembled based on *in vitro* observations, which have been central to our understanding of how the clock is regulated at the molecular level (Figure 3).

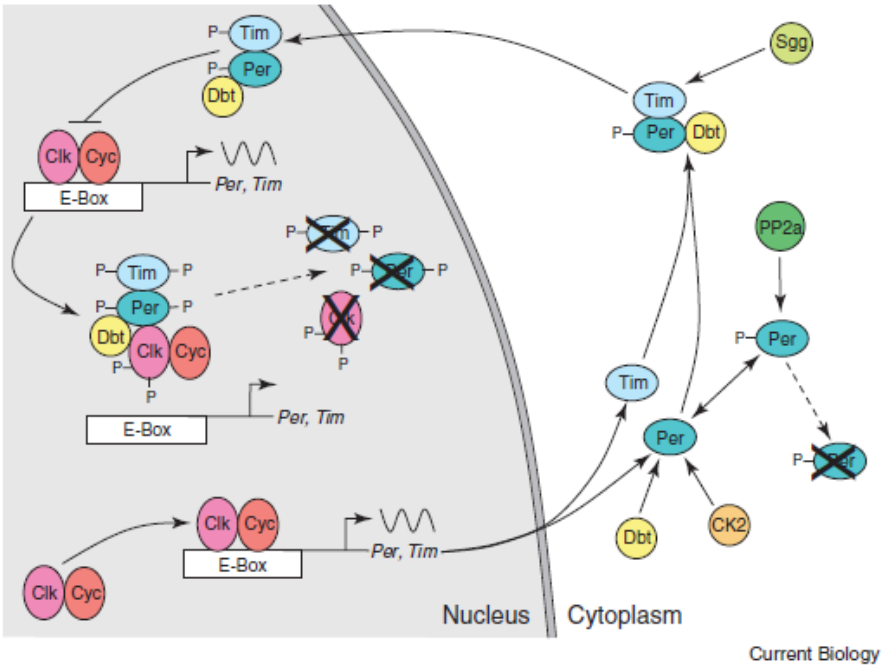


Fig. 3. The core molecular clock in *Drosophila melanogaster.* Model of the PER/TIM feedback loop (illustrated by Hardin, 2005)

In flies, the molecular-genetic basis of circadian time-keeping may rely on a two interconnected transcriptional – transitional feedback loops which control expression of several genes, that can generally be divided into groups according to the molecular nature of their products (as transcription activator, transcription repressor or protein degradation player) and the changes created on their own steady – state levels (reviewed by Hardin, 2005) along the day. More in details, the flies core clock is composed of a positive loop – rhythm driving factors, consisting of two transcription factors CLOCK and CYCLE proteins (Allada *et al.*, 1998; Rutila *et al.*, 1998) that contain a basic helix – loop – helix (bHLH) DNA binding domain and a PER –ARNT- SIM (PAS) dimerization domain, that activate transcription of many genes, including *period* (*per*) and *timeless* (*tim*), and a negative loop elements, the PERIOD (PER) and TIMELESS (TIM) proteins, that acts as transcription repressor in the molecular cycle (Dunlap, 1999). Generally, both *per* and *tim* mRNAs and proteins, exhibit rhythmic abundance, with peaks resulting respectively during the early and the later part of the night, reflecting the corresponding behavioral rhythm (reviewed by Sharma,

2003). On the other hand, *clock* genes show cyclic expression too, with mRNA and protein level peaking early in the morning, whereas *cycle* is constantly expressed. In wild type flies, to initiate the negative loop, CLK heterodimerizes with CYCLE in the middle of the day, binding an E-box regulatory elements in the regulatory regions of *per* and *tim,* thereby activating their transcription, which peaks its level early in the night (Fig. 3). PER and TIM proteins interact to form an unstable PER:TIM heterodimer and translocate into the nucleus, without accumulating till late evening, when they peak their level. After a delay, due to phosphorylation of *pe*r dependently upon two kinases, DBT and CASEIN KINASE 2 (CK2), leading to its degradation, till stabilization of phosphorylated *per* by binding either to *tim* or PROTEIN PHOSPHATASE 2a (PP2a), which is supposed to remove the phosphates added to PER (reviewed by Hardin, 2005). In the meanwhile, as TIM levels increases, the remaining complex TIM-PER-DBT translocates to the nucleus upon SHAGGY (SGG) – dependent TIM phosphorylation, which, in concert with CK-2 dependent PER phosphorylation, lead to their transport into the nucleus (reviewed by Hardin, 2005). Recently, it has been argued that PER and TIM can enter into the nucleus as dimer complex formed by protein – protein interaction without any DNA – binding domains, due to PER capacity to associate with other transcription factors to negatively regulate its own transcription (reviewed by Sharma, 2003) and to act as transcriptional repressor, specially that *tim* sequence lacks of DNA-binding domain and PAS domain (reviewed by Shirasu *et al.*, 2003). Once in the nucleus, TIM-PER-DBT complex binds to the CLK-CYC heterodimer, inhibiting further *per* and *tim* expression by preventing the DNA binding to E-box of CLK-CYC dimer complex (Lee *et al.*, 1999; reviewed by Hardin, 2005), thus repressing CLK-CYC activity. In the nucleus, DBT phosphorylation leads to PER and CLK degradation, while TIM destabilization is triggered via a light – mediated pathway, by tyrosine phosphorylation. Consequently, the accumulation of the hypophosphorylated CLK, in addition to a PER-TIM heterodimer decays, trigger heterodimerization of CLK with CYCLE and the starting of *per* and *tim* transcription again*;* thus the cycle repeats itself, which resets the mechanism of circadian clock (reviewed by Hardin, 2005).

Another identified component playing an important role in the second transcriptional – translational interlocked feedback loop is the transcription factor encoded by *vrille* (*vri*) and *par domain protein 1 ε* (*pdp1ε*) genes. Both seems to regulate directly *Clk* transcription by specific binding to *Clk* promoter elements and functions as respectively repressor and activator elements of *Clk* transcription (Fig. 4) (Cyran *et al.*, 2003; Glossop *et al.*, 2003; as reviewed by Hardin, 2005).

The CLK-CYC heterodimer drive expression of *vri* and *pdp1ε* by binding to the E-Box sequence in the promoter region, trigging their highly transcription, respectively by late day and early night (Cyran *et al.*, 2003; Glossop *et al.*, 2003; as reviewed by Hardin, 2005), with *vri* mRNA cycling in phase with those of *per* and *tim*. Once VRI accumulates, it tends to bind the VRI/PDP1ε box regulatory elements and repress CLK transcription till VRI displacement form the (V/Pbox) by PDP1ε, and consequently de-repression of CLK transcription (Cyran *et al.*, 2003). In addition to the effect on the central clock's components, *VRI* affects the output system through the peptide *PDF* (reviewed by Williams & Sehgal, 2001), that is strongly suggested to function as a circadian transmitter.

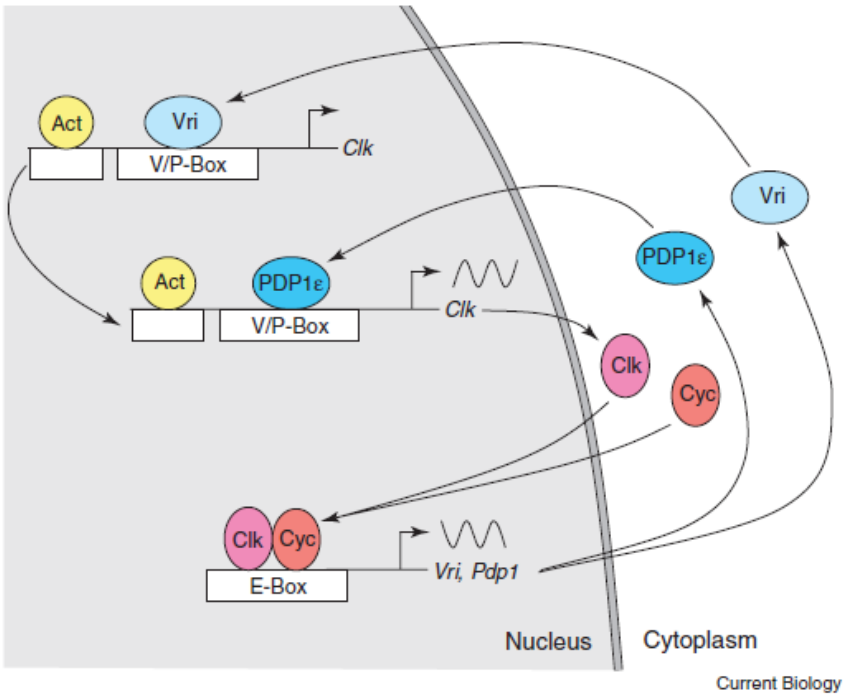


Fig. 4. The core molecular clock in *Drosophila melanogaster.* Model of the CLK feedback loop (illustrated by Hardin, 2005).

Molecular genetic studies in *Drosophila* identify some additional clock components. These circadian regulatory molecules act to refine the transcriptional/translational feed-back loops. Light changes the levels of these additional clock components, resetting the clock to different time of day. In *Drosophila*, the entrainment of the circadian clock relies on the degradation of TIM in response to light. Cryptochrome (*CRY*) is the major mediator for the rapid light-induced degradation of TIM, which has been demonstrated to be mediated by a ubiquitin-proteasome

pathway into neural and peripheral clock cells (reviewed by Dubruille & Emery, 2008). Light activation of CRY, according to a light dependent rhythm (Emery *et al.*, 1998), permits its binding to TIM (Ceriani *et al.*, 1999), which in turn leads to TIM degradation by a tyrosine kinase phosphorylation. Thus, TIM degradation through the CRY photoreceptor, in addition to its ability to synchronize the internal clocks to the 24 h cycle of sunlight, plays a relevant role in shortening or advancing the phase of the clock, according to the time of exposition to light - pulses (during early or late night) (reviewed by Allada & Chung, 2010) and indirectly to the levels of *tim* mRNA (reviewed by Hardin, 2005).

1.2.3 Multiple tissues express clock genes

In flies, concerns regarding the overall organization of the circadian system trigger the characterization of several essential clock genes; and the analysis of their expression patterns gave evidences that the molecular components of the circadian clock are largely conserved outside the central master in a broad range of organs (reviewed by Giebultowicz, 1999). This, together with relevant researches investigating clock molecule cycle in various biological functions, such as metabolism, reproduction or excretion, without focusing primarily on the central clock, reinforced evidences that circadian oscillators may function by maintaining coordinated physiological processes, in a tissue – autonomous fashion (Giebultowicz, 1999; Brown & Schibler, 1999). Although many advances have been made recently in enlarging our understanding of the peripheral clocks and their role in circadian timing, many questions remain unanswered yet, regarding how many clocks oscillate in pluricellular animals, such as insects, how much precise influence have the master clock over fly peripheral oscillators, in the sense if they do require input from the nervous system, and in which manner such peripheral ensemble of clocks ensures the internal temporal organization of the organism.

In insects, the first organs that have been revealed to have an independent pacemaking function and to be directly entrained by environmental signals is the testes – vas deferens complex in the gypsy moth, *Lymantria dispar* L., that has shown a light entrainable sperm release rhythm when investigated *in vitro* (Giebultowicz *et al.*, 1989). In fact, the fertility of the sperm seems to be dependent on the occurrence of two essential steps, which rhythms register two peaks occurring in the evening and morning and respectively consisting of the release of sperm from the testis into the upper vas deferens (UVD) followed by the accumulation of the sperm in the seminal vesicles.

Interestingly, such circadian rhythmicity has been revealed to persist under constant darkness and to happen in correlation with glycoproteins release from the apical portion (Giebultowicz *et al.*, 1989). In fact, further studies confirmed the importance of the circadian coordination between either the sperm maturation process or the glycoprotein's secretion, as a guarantee of the efficiency of the male flies' reproductive system (Bebas *et al.*, 2002). Importantly, the temporal relationships of the *per* clock gene mRNA and PER protein expression rhythms within cells surrounding peripheral tissues testes and vas deferens (Gvakharia *et al.*, 2000; as reviewed by Giebultowicz *et al.*, 2001) are of great impact on such coordination. These findings were supported by persistent cycling of the *per* clock gene and its proteins in fly pupae ring gland, when cultured *in vitro* (Emery *et al.*, 1997; as reviewed by Giebultowicz, 2001). On the other hand, and mainly by high – time resolution experiments using *per* – driven rhythmic luciferase activity once a promoter region fused to luciferase – encoding sequences, enormous progress has also been made in determining either the *in vivo* regulatory pattern of the *Drosophila* clock gene *period (per*) or in providing a powerfully reproducible measure of its transcriptional oscillations in organs that were analyzed *in vitro* (as cited by Hardin, 2005). From such kind of experimental systems, a wide range of self-sustained and photosensitive oscillators in *Drosophila* peripheral tissues have been reported in renal tubules and rectum (Giebultowicz *et al.*, 2000), chemosensory structures located at the level of antennae, proboscis, wing margin and legs (Plautz *et al.*, 1997; as reviewed by Giebultowicz, 2001). In the meanwhile, the list has been extended to an additional type of peripheral organs, that were promoted as autonomous functioning oscillator clocks, which potentially oscillate independently from the central clock. Recently, Tanoue *et al.* (2004) have shown that components of the olfactory signal transduction can function as an autonomous pacemaker for olfaction rhythms, even in absence of central circadian regulation, due to targeted lateral neurons ablation, provided the ablated antennal neuron in *cyc* (*01*) flies is rescued.

The same situation seems to prevail in *in vitro* cultured *Drosophila* testes, where a rhythmic activity of either *per* or *tim* luciferase reporters has been registered. Another piece of evidence, suggesting a high degree of autonomy of peripheral pacemaker in flies, comes from examining the role played by fat body tissue in modulating the daily rhythm of feeding behavior activity (Xu *et al.*, 2008). A number of metabolic genes, has been determined through microarrays screens, to be cyclically expressed at the level of metabolic tissues, driven by a local circadian clock (Xu *et al.*, 2008). In fact, inactivation of clock functioning by clock gene knockouts techniques have reportedly triggered symptoms of metabolic dysfunction, such as increased food consumption (Xu

et al., 2008), as previously demonstrated in mammals (Turek *et al.*, 2005; Colles *et al.*, 2007; Bray & Young, 2007; as cited by Xu *et al.*, 2008).

At the molecular level and contrary to the case of mammals, it has been suggested in invertebrates, and more precisely in flies, that peripheral oscillators respond to light resetting signals due to the photoreceptor role of CRY (as reviewed by Hardin, 2005). At the peripheral level, such autonomous photoreceptor cell seems to have predominately the ability to convey light information to oscillator without being completely integrated in the core clock components. In fact, in the cry^b mutant flies, antennal clock neurons registered an impaired free – running rhythms, combined to reduction till disruption of clock driven olfactory rhythms (Krishnan *et al.*, 2001; as cited by Merlin *et al.*, 2007), which restoration is dependent on antennal functioning neurons rescue (Tanoue *et al.*, 2004). Moreover, the same mutation (cry^b) triggers dysrythmia of the core clock genes *per* and *tim*, in other tissues such as fly organs (Levine *et al.*, 2002) or Malpighian tubules (Mts) (Ivanchenko *et al.*, 2001), supporting CRY as dispensable within the core clockwork. All these findings support massively the specialized role for circadian oscillators in mutual regulation of metabolism or physiological processes and circadian rhythms, in order to provide effective tissue's response and adaptation to environmental signals.

1.3 The Honey bee: an interesting model for understanding mechanisms of life history transitions

1.3.1 Honey bee as a model system

Honey bees (*Apis mellifera* L.) are eusocial insects that live in large colonies consisting of some 20,000 up to 40,000 female workers (sterile or having low reproductive potential), and 200 to 300 male drones, and a queen (extremely fecund), interacting all together according to an elaborate communication system (Wilson, 1971) that allows them to coordinate efficiently each aspect of their life. Social insects, and bees particularly, could be considered as an interesting model system that offers an opportunity to study a number of fundamental aspects of the evolution of sociality with an integrative focus on the molecular, physiological and endocrine processes involved in their life history transitions and/or their adaptive responses to the environmental context in which they

live. This appears to be especially true for this category of social insects, that is assumed to exhibit a temporal polyethism, allowing the adults to move through successive behavioral stages in an age-related fashion, which, in addition to a pluriannuality in reproduction, provided a base for developing an arsenal of researches that led simultaneously to explore deeply the ecology, natural behavior and phylogeny of these insects (reviewed by Elekonich & Roberts, 2005). Moreover, honey bee figures within the list of organisms which genomes have been sequenced and along with a panel of related encoded proteins are largely represented in a abundant literature (reviewed by Denison & Delpech, 2008).

Based upon the fact that honey bees undergo a natural switch from a phase when they work in the hive to another when they forage outside, with behavioral rhythms that, in addition to be primarily age-determined, are generally also socially mediated, a large interactive research has been performed, using “the single cohort colonies” (reviewed by Elekonich & Roberts, 2005) deriving from the original typical colony. These single cohort colonies are based on discrimination of specimens (typically age *vs* behavior) that allows the collection and observation of precocious foragers (young workers) or reverted nurses (typical aged bees) obtained as a consequence of an unbalanced colony age demography (Fig. 5) (Robinson *et al.*, 1989; Huang & Robinson, 1992; Giray & Robinson, 1994; as cited by Elekonich & Roberts, 2005); investigation on these colonies has shed light on workers-worker interaction determinism of physiological and genetic mechanisms involved during the behavioral transition (Huang & Robinson, 1992).

Honey bee has shown also to be a promising model system for researches that, through useful manipulations, succeed in training the foragers to specific feedings stations and time (Von Frisch, 1964), helping to establish time memory properties of honey bee worker. Others practices were made to obtain colonies characterized by mixed genotypes, that facilitate investigation on genotypic influences on individual versus colony traits (Page *et al.*, 2000), in addition to various pharmacological treatments (Ben-Shahar *et al.*, 2003) or pheromones analogs (Grozinger *et al.*, 2003) that allowed a major efficiency in bee behavior and physiology dissection (reviewed by Elekonich & Roberts, 2005).

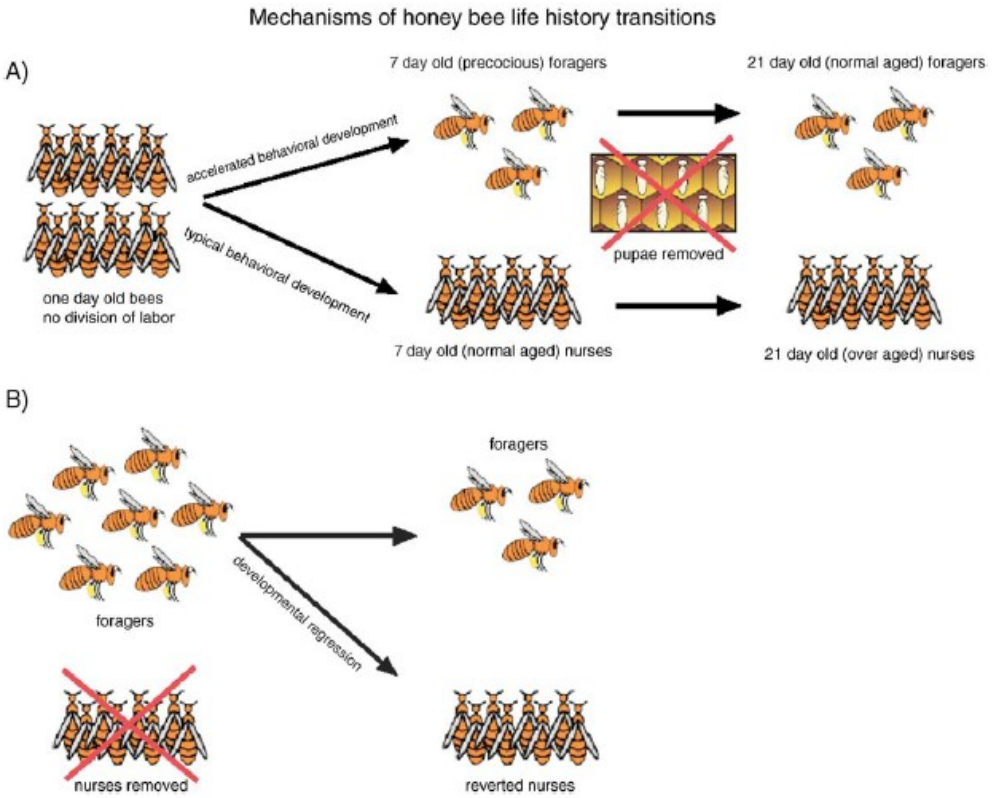


Fig. 5. Behavioral development in a Single Cohort Colony (SCC). (A) The typical SCC is obtained from the collection of newly emerged bees (1 day old). It is assumed that only the 10% will follow a precocious development without any connection between the age and the behavior. Thereby, observations are made in presence of a group consisting of a number of young (normal age) nurses and young (precocious) foragers, and later old (over-aged) nurses and old (normal aged) foragers, just in absence of additional newly emerged bees. (B) Reversion SCC, consisting in removing nurses from the group which trigger the reversion of foragers to brood care. Thus, observations will be made in the presence of workers that experienced foraging, divided into foragers reverted to an earlier stage (nursing work) and old (normal aged) foragers (adapted from Elekonich & Roberts, 2005).

1.3.2 The social clock of honey bee

1.3.2.1 Social influences on the ontogeny of circadian rhythm

Such as a representative of a very advanced social insect, each honey bee emerges and develops inside a social environment and in presence of conspecific individuals belonging to all life stages (including eggs to adult workers) which, through an elaborated ways of communication by either direct (trophallaxis) or indirect (pheromones, vibratory, visual or acoustic signals) contact, succeed considerably in driving characteristics of the circadian rhythm (phase, expression or development) of the single individual bees. Such a social entrainment has been deeply studied by many researchers (Southwick & Moritz, 1987; Moritz & Sakofski, 1991; Frisch & Koeniger, 1994; Moritz &

Kryger, 1994) and seems to influence the social behavior in a functionally significant way that guarantees colony efficiency.

In the same order of ideas, and contrary to other insects, honey bee expresses circadian rhythm only after adult emergence (Moore, 2001), according to a precise postembryonic ontogeny in the development of such a rhythm. In their natural environment, the newly emerged bees remain inside the nest, without any or limited contact with the external environment, and specialize in taking care of the brood and in other "in-hive" activities (like hive maintenance) around the clock, with an attenuated molecular and behavioral rhythm (Crailsheim *et al.*, 1996; Moore *et al.*, 1998). After the first 2 to 3 weeks of adult life, the worker shift to foraging for nectar and pollen outside the hive, according to a strong circadian rhythm which is necessary for the sun compass navigation, for the recruitment waggle dance and for the time memory regulating the timing of foraging to the maximum nectar and pollen reward availability (Fig. 6) (reviewed by Moore, 2001). Interestingly, the nurse bees, which are usually active around the clock when examined inside the colony, have been shown to exhibit a strong circadian oscillations, either in locomotor activity or gene clock expression, once they have been isolated individually from the brood (Shemesh *et al.*, 2007, 2010; as reviewed by Eban–Rothschild & Bloch, 2012) or under constant conditions in the laboratory (Eban-Rothschild *et al.*, 2012), which has been incongruent with the hypothesis suggesting that the circadian clockwork is not functional at young stage of life. Moreover, this consistent behavioral plasticity has been associated with a functional molecular feedback loop in nurse clock, that tends to free run under constant conditions, supporting the idea that the ontogeny of the circadian rhythm in honey bees seems to be socially fine tuned and endogenously defined (Toma *et al.*, 2000; Bloch *et al.*, 2001, 2003, 2004; Shimizu *et al.*, 2001; as reviewed by Bloch, 2010).

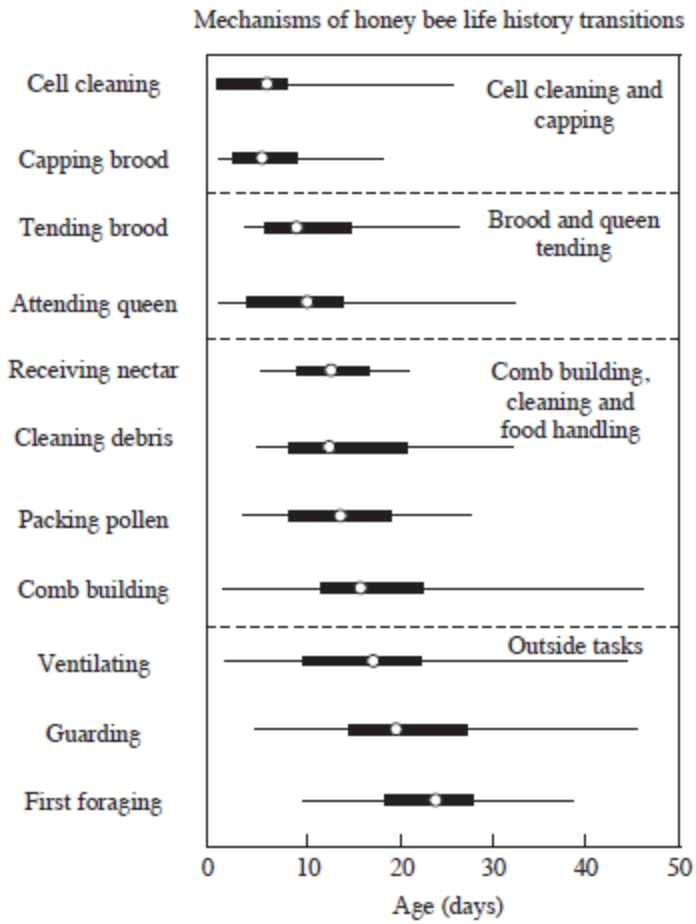


Fig. 6. Age-related task performance ("Temporal polytheism") by worker honey bees (adapted from Winston, 1987; by Elekonich & Roberts, 2005).

In the meanwhile, a wide range of researches aims at identifying whether the social signals that generally mediate the task-related behavioral development could be of efficiency according to the social context to which young workers will be adapted, either at the molecular or behavioral level. To answer such question, many intra- and inter-individual manipulations have been made. Neither the use of biogenic amines or some of their antagonists (reviewed by Bloch *et al.*, 2009) nor the removal of their source of secretion (*corpora allata*) appeared to affect the age at onset of rhythmicity; this trigged the hypothesis that Juvenile hormone (JH) and/or octopamine could be involved in some non circadian functions, which are originally settled by other *period* mRNA expressing cells, that are diverse from those cells influencing the diurnal locomotor activity (Bloch, 2010). In the case of nurses that have been monitored individually in the laboratory, soon after having experienced the colony environment after emergence, synchronization with social factors is perhaps the most predominant element influencing the ontogeny of circadian rhythms, as well as the overall locomotor activity or its free-running period (Meshi & Bloch, 2007; Eban–Rothschild

et al., 2012; as reviewed by Eban–Rothschild & Bloch, 2012). Even if little is known about the mechanisms underlying the influence of the post embryonic development of internal circadian rhythms, Eban–Rothschild & Bloch (2012) have been more supportive of the hypothesis that rely the delay in overt circadian rhythm, in the case of isolated young bees outside the colony, to the effect of in-hive environmental conditions, including either the social (as pheromones) or the microenvironment (such as temperature etc.) factors rather than to others (such as physical contact with conspecifcs) or the light dark illumination regime.

1.3.2.2 Social modulation of plasticity in the expression of circadian rhythm

It is well known how honey bees show a specific plasticity in expressing their circadian rhythms, enabling them to switch from working around the clock to diurnal foraging activity with robust circadian rhythms, according to the temporal division of labour (reviewed in Bloch, 2009, 2010; Eban–Rothschild & Bloch, 2012). Such task-related plasticity in the circadian clockwork, that has been well defined as modulated by the social environment, is suggested to be functionally relevant and serves as guarantor of maximal efficiency of the whole colony (Bloch, 2010; Bloch & Grozinger, 2011). In natural conditions, relevant results have already suggested that, while the presence of brood could be involved in the attenuation of the expression of workers circadian rhythms (Shemesh *et al.*, 2010) by acting probably as a masking agent, the isolation of the young nurses from any colony life, either in the laboratory or in broodless comb or outside the hive, promotes the diurnal patterns of expression in locomotor activity and also clock genes expression. Likely, previous studies, that adopted social manipulations leading to uncouple the age and tasks to perform, have shown the reversal of honey bee behavioral rhythms, which linked strongly the synchronization of the molecular clockwork more to the task than to age (Bloch & Robinson, 2001; Bloch *et al.*, 2001). Thus, it seems that at least the brood presence plays an important role in strongly modulating such plasticity in circadian clockwork and, interestingly, it has been demonstrated that the brood social synchronization may strongly be mediated through sensory signals whose detection involves the antennal flagella of the worker (Nagari & Bloch, 2012). The evidence of the involvement of the social signals in modulating plasticity in the circadian rhythms is provided also by other eusocial insects, including ants and bumble bees (Ingram *et al.*, 2009; Yerushalmi *et al.*, 2006). For example, bumble bees follow a similar chronobiological plasticity, overall behavior and molecular patterns, that is highly modulated by size–related division of labor

rather than age-dependent one (Yerushalmi *et al.*, 2006). On the contrary, harvester ants are more unlikely to exhibit an age–related polyethism, associated with developmental regulation of clock genes expression, promoting some similarities between them and honey bees (Ingram *et al.*, 2009). So far, such findings could validate the theory of the "internal temporal order" to which the colony macro-environment of such social groups is associated (cited by Teixeira *et al.*, 2011). Such remarkable natural plasticity in circadian rhythm may represent a conserved selective toolkit adopted by those species and translated through individual adoption of the right endogenous temporal system that occur following a precise phase-relationship, in synchrony with those expressed by others of the community, to guarantee a functional coordination of the "Super–organism" (Moritz & Fuchs, 1998) and consequently the maximal survival in an ecological context.

1.4 Thesis objectives

The aim of the thesis is to emphasize on the study of the circadian regulation of flight muscle tissue in honey bee *Apis mellifera*. Circadian clocks synchronize behavior and physiological functions to the fluctuating environment (Dunlap *et al.*, 2004). In social insects, the circadian clock is highly influenced by the social interactions between individuals within their colony. In honeybee for example, is thought that the behavior and circadian clockwork exhibit generally profound context-dependent plasticity, enabling adjustments to rapid changes in the social environment (Bloch, 2010). However, little is known about the function and mechanism of such interplay between social needs and sensitivity of the circadian system, in tissues of the body periphery. Due to the parallels between the circadian mechanisms of honeybee and mammals, I hypothesize that a peripheral clocks regulate flight behavior in worker bees. In Chapter 2, I examine whether the main core clock gene undergo differences in the circadian regulation in the flight muscle tissue, from young nurses and adult foragers collected from field colonies under natural LD conditions. I found that the core clock genes are circadianly expressed in a period-dependent fashion in flight peripheral tissue of both workers. Nevertheless, the temporal gene expression differed in the wave form and phase between our witnesses of the transition from nursing to foraging behavior. Consequently, we assessed the endogenous nature of such peripheral clock, when exposed to different cycle of continuous darkness, at different key ages, over the honeybee life span. Additionally and under the same conditions, we verified our hypothesis concerning the fact that

the flight muscle tissue might contain a circadian clock which regulates itself the temporal expression of genes involved in relevant metabolic activities. Comparing the clock gene expression profile between brain and flight muscle tissue at each age sampling, add functional insights related to the nature of the coordination that happen between oscillators, throughout the developmental related circadian regulation of the flight muscle in honeybee. This study sheds new light on the molecular dynamics and social regulation of context-dependent plasticity in the organization of the circadian clockwork of the honeybee.

2. FIELD RESEARCH

2.1 Introductory notes

Circadian rhythms govern the behavior, physiology and metabolism of living organisms (Hall & Rosbash, 1993). These circadian rhythms are driven by a cell-autonomous daily timekeeping mechanism, that allows organisms to adapt to the changing environment and thereby optimize their survival. The key features of circadian clock are its ability: i) to synchronize (entrain) to external daily rhythms of light, temperature and other environmental cues, and ii) to persist (free-run) when placed in constant conditions with close to a 24h (circadian) period.

Genomic analysis of behavioral rhythm in flies has elucidated the molecular and cellular components of the core clock, giving better understanding of the tools by which the molecular circuitry underlying circadian function are conserved in other organisms. In *Drosophila*, the molecular basis for these rhythms is proposed to consist of molecular feedback loops, which comprise several core clock genes, such as *period (Per), timeless (Tim), clock (Clk) and cycle (Cyc).* These genes encode PER, TIM, CLK and CYC proteins, respectively, which orchestrate changes in their own steady-state levels and that of their mRNAs. More precisely, the two transcription factors CLOCK (CLK) and CYCLE (CYC) form heterodimers and activate transcription of *Per* and *Tim* genes by binding to their E-boxes in their promoter regions. Resulting PER and TIM proteins bind to each other and enter to the nucleus, where they repress their own CLK:CYC complex induced transcription, allowing the cycle to begin anew. The stability and ability to accumulate to levels that are functional of PER and TIM is extensively regulated by CK1ε protein DBT and Casein Kinase 2 phosphorylation, that promote their degradation. The CLK:CYC heterodimer also regulates the expression of two transcription factors Vri and Pdpε, that form the second feedback loop by regulating the transcription of *Clk*. Simultaneously, molecular output generated from the central oscillator to overt rhythms is regulated via the induction of rhythmic transcription of an array of Clock controlled genes (ccgs) such as *pdf*, takeout or *lark*.

Although the anatomical site of the central circadian clock in insects is the brain area, recent studies about spatial distribution of clock gene expression revealed the presence of peripheral oscillators existing in many tissues in fly bodies. While a persistent *Per*-driven bioluminescent

oscillations occurred *in vivo* in the compound eyes, antennae, proboscis, wings, legs in *per-luc Drososphila* (Plautz *et al.*, 1997), other fly's tissues as the malpighian tubules in adults (Giebultowicz & Hege, 1997; Hege *et al.*, 1997), the ring gland in pupae (Emery *et al.*, 1997), the alimentary tract, fat bodies, ovaries or testes (Liu *et al.*, 1988) have been reported to free run and to be directly entrained by light when isolated *in vitro*. Taken together, these findings attest that such peripheral tissue clocks have the potential to function independently from the central clock in the brain, generating local clocks controlling tissue-specific functions. To our knowledge, the organization of the circadian system in social insects, such as bees, is still in its infancy and the possible presence of widespread oscillators outside the brain has never been considered yet.

Bees have specific biological clocks that function in time memory, enabling visiting flowers at time of maximal nectar and pollen availability, time-compensated sun-orientation and recruitment waggle dance (Von Frisch, 1967). The biological clock used in these behaviors of foragers is considered to be based on a circadian system and its endogenous nature has been shown repeatedly for general locomotor activity in individual honey bees (Toma *et al.*, 2000; Bloch *et al.*, 2001, 2004; Rubin *et al.*, 2006; Shemesh *et al.*, 2007, 2010), which rhythm persist ("free-run") under constant dark (DD) conditions with a period of about 24h. Because bees are social organisms that live in a complex and highly organized society, notion of timekeeping mechanisms should be correlated with division of labor that organizes workers, leading to occurrence of behavioral or physiological processes according to the right moment and social context. In field colonies, honey bee workers showing evident temporal polyethism evolve across their life span, from nursing brood around the clock, with no circadian rhythm during the first 1 to 2 weeks of adult life, to foraging activities later (typically > 3 weeks of age), with a strong circadian rhythms (Kaiser & Steiner-Kaiser, 1983; Moore *et al.*, 1998; Sauer *et al.*, 2003). Indeed, and altough the studies on molecular basis of circadian rhythm in social insects have only started recently, various studies already report how the age related division of labor is highly associated with the differences in the mRNA levels of the main core clock *period* gene in the brain (Toma *et al.*, 2000; Bloch *et al.*, 2001, 2004). From self–sustained colonies, *Per* expression tend to oscillate at its highest during the night in brain of rhythmic foragers, contrary to their young sisters that typically do not show any circadian rhythms in clock genes expression (Bloch *et al.*, 2001; Bloch & Meshi, 2007; Meshi & Bloch, 2007) exceptionally till 7 days old, if they do not experience any social environment influences (Shemesh *et al.*, 2007, 2010). This evidences make tempting to investigate

how such circadian system behaves outside the brain in honeybees. Indeed, evaluating such scenarios would be a good starting point for understanding if the central oscillator regulates cellular processes in different tissues or whether there are specific rhythmic functions of cellular processes, driven by functionally separable oscillators, organized with respect to internal temporal order of such social insects and their age related development needs.

Such investigation can be interestingly undertaken in the case of honeybee's muscle tissue, which undergoes related molecular and biochemical lifetime changes according to the adult behavioral transitions and the consequent physiological capacity needs (reviewed by Roberts & Elekonich, 2005).

The field part of this research, whose results are here reported, simultaneously examined the 24h mRNA expression profiles of two main core clock genes, *Per* and *Cry*, and the myogenic factor gene *Nautilus* (*Nau*) involved in honeybee muscle metabolism, every 4h in brains and flight muscle isolated from individual workers in field–collected colonies under natural LD conditions. The main objective of the present study was to provide for the first time a comparative description of the expression patterns between foragers and their nurse sisters at the central and flight muscle peripheral level and to verify any correspondence to differences in locomotor activity.

2.2 Materials and methods

All the field experimental part of the research exposed in this report, and the consequent lab work strictly correlated to it, has been carried out at the Department of Biology of the University of Puerto Rico, Rio Pedras campus, where I had the possibility to spend a fruitful study and training period, under the guidance and scientific responsibility of Prof. Tugrul Giray, subdivided in two experimental periods, for totally 6 months between November 2011 and June 2012.

2.2.1 Bees

Triple cohort colonies were kept according to standard beekeeping techniques in the local greenhouse facility of the Department of Biology of the University of Puerto Rico. Climatic

conditions during the whole experiments where characterized by the fairly stable temperature (maximum=30°C; minimum =24,8°C) and the irregular rainfall. The Africanized bees used as source colony for the experiments were derived from a mixture of African race (*Apis mellifera scutellata* Lepeletier) and several European races, typically diffused in Puerto Rico. They were collected in November 2011, a time of the year that corresponds to approximately 11h light and 13h dark, at longitude 66.5 W, latitude 18.15 N (San Juan, Puerto Rico). To determine whether results obtained from the free-flying colony reflect accurately the functioning of the circadian system under natural environment, care was taken to house the hive in a specially designed tent (made of an opaque fabric 3x3x3 m) (fig. 9), so exposure of foragers to light was limited to day (when they flew outside) and to darkness only during the night (when the hive was hidden by the tent), while nurses were mostly still experiencing constant dark environment (inside the hive). In the meanwhile, all manipulations and collections were made under dim red light, which bees cannot distinguish (Von Frisch, 1967).

The bees collected for mRNA analysis were identified according to standard criteria (Moore *et al.*, 1998; Bloch & Robinson, 2001; Block *et al.*, 2004). Nurse bees seen with their heads inside honeycomb cells containing larvae or eggs were given a paint spot on the dorsal surface of the thorax. On the other hand, foragers were marked by another color, when returning to the hive with a load of pollen in their pollen baskets. The obstruction of the hive entrance by an eight-mesh pitfall was useful for foragers identification. During the whole experimental activity, each sample (N = about 20 bees/time point/type of worker) was collected at 6 different time points (00 a.m., 04 a.m., 08 a.m., 12 a.m. 16 p.m., 20 p.m.) during 24h. The frequency (every 4h) and duration (24h) of sample collection was required to establish the 24-hr repeating oscillation pattern of circadian mRNA expression. Bees for analysis were directly introduced in the liquid nitrogen and stored at -80°C until brain and flight muscle dissection.

2.2.2 Brain and flight muscle dissection

After storage, the different tissues were isolated and dissected under dry ice and kept frozen during the entire dissection. Brain dissection consisted of removing compound eyes, ocelli, hypopharyngeal glands and any other glandular tissues whereas the flight muscle was dissected by dividing the frozen thorax in half by scalpel and eliminating the cuticle, in order to isolate the two

pieces of ventral-dorsal thoracic muscle accurately. Subsequently, all intact brains and muscles were stored individually at -80°C until mRNA analysis.

2.2.3 RNA isolation and cDNA synthesis

For each time point sample, total RNA was isolated from a 2 replicated pools of 3 bee brains each (using the RNeasy Micro kit; Quiagen, Valencia, CA) and 2 others of 3 flight muscles each (using the RNeasy Fibrous Mini Kit, Quiagen, Valencia, CA), according to the manufacturer's instructions. Prior to cDNA synthesis, integrity of RNA was determined using the RNA 6000 Pico Lab-on-a-chip kit and the Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA), according to the manufacturer's instructions too. The concentration of total RNA was performed using a NanoDrop ND 1000 spectrophotometer V 3.5.2 (NanoDrop Technologies, Wilmington, DE).
First strand cDNA was synthesized from 50 ng of total RNA, from each brain and flight muscle sample (described above) using the TaqMan Reverse Transcription Reagents (Invitrogen, CA) including random hexamer Primers, according to the manufacturer's instructions. The reaction volume for each sample was corrected to 20 µl using nuclease free water (Quiagen, CA) and stored at -20°C.

2.2.4 Real-time quantitative RT-PCR

We measured mRNA levels with real time quantitative reverse transcription PCR (qRT-PCR) using the Mx3005P real-time PCR System (Stratagene – Palo Alto, CA) and ITaq Universal SYBR Green Supermix (BIO-RAD, CA). From the literature, a list of core clock genes (*Per* and *Cry*) and the myogenic factor gene (*Nau*) involved in muscle metabolism in honeybees was established. GenBank (NCBI) – published honeybee sequences and PrimerQuest (Integrated DNA technologies, http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/) software were used to design the specific primers of candidate genes (Tab. 2). BLAST (Basic Local Alignment Search Tool) (www.blast.ncbi.nlm.nih.gov) and Oligo Analyzer (Integrated DNA technologies, as above) software were used to respectively ensure the sequence specificity of primers and prevent self-complementarities and self dimmers stability. Each 20 µl reaction contained 2 µl cDNA (0,55 ng/µl), 3 µl forward and reverse primer mix (2,5 µM), 10 µl ITaq Universal SYBR Green Supermix (BIO-RAD, CA), and 5 µl nuclease-free water (Quiagen, CA). For each qRT-PCR assay, a standard

curve was generated using twofold serial dilutions of a pooled cDNA. Each well of the plate was produced in duplicate in the Stratagene MX3005P machine. The amplification thermal profiles consisted of one cycle of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min, with a continuous fluorescence measurement. Target genes mRNA levels were measured by determining the cycle number at which amplification detection threshold was reached (CT). Transcript abundance was determined relatively to *AmRPS5* (ribosomal protein 49) selected as internal reference gene, according to its greater stability in honeybee brain and flight muscle tissue over time (Lourenço *et al.*, 2008).

2.2.5 Statistical analysis

Daily variation of mRNA expression was statistically analyzed using repeated measures analysis of variance (ANOVA) with Graph Pad Prism software Version 5.0 for Windows (Graph Pad software, San Diego, California, USA, http://www.graphpad.com). The values of the relative expression of mRNA are presented as the mean ± SEM. A value of $P<0.05$ was considered significant.

2.3 Results

The whole previously described field part of the research allowed us to compare, by real-time quantitative RT-PCR, the expression patterns of two core clock genes, *Per* and *Cry* and the core clock controlled gene (CCG) *Nautilus (Nau)*, in both forager and nurse honeybees, under natural environmental conditions, in a free flying colony. The rhythmic transcription of *Per* and *Cry* was investigated in both the brain and the flight muscle of the sampled bees, while the mRNA expression of the all 5 aforementioned muscle metabolic genes was only detected in the flight muscle tissue.

2.3.1 Flight muscle tissue expresses robust circadian oscillations for circadian genes

The repeated measures ANOVA (n=6) revealed a significant variation in expression levels over time for the two core clock genes *Per* and *Cry* ($p<0.05$ in nurses, for both the genes, and respectively $p<0,001$ and $p<0,5$ in foragers, Tab.1) in the flight muscle of both nurse and forager honeybees. On

the other hand, and prior to the onset of the dark phase of the photoperiod at ZT16, the peak expression of *Per* was at its highest in the foragers and interestingly at its lowest in nurses, under a 11:13 – h light – dark photoperiod (Fig. 7).

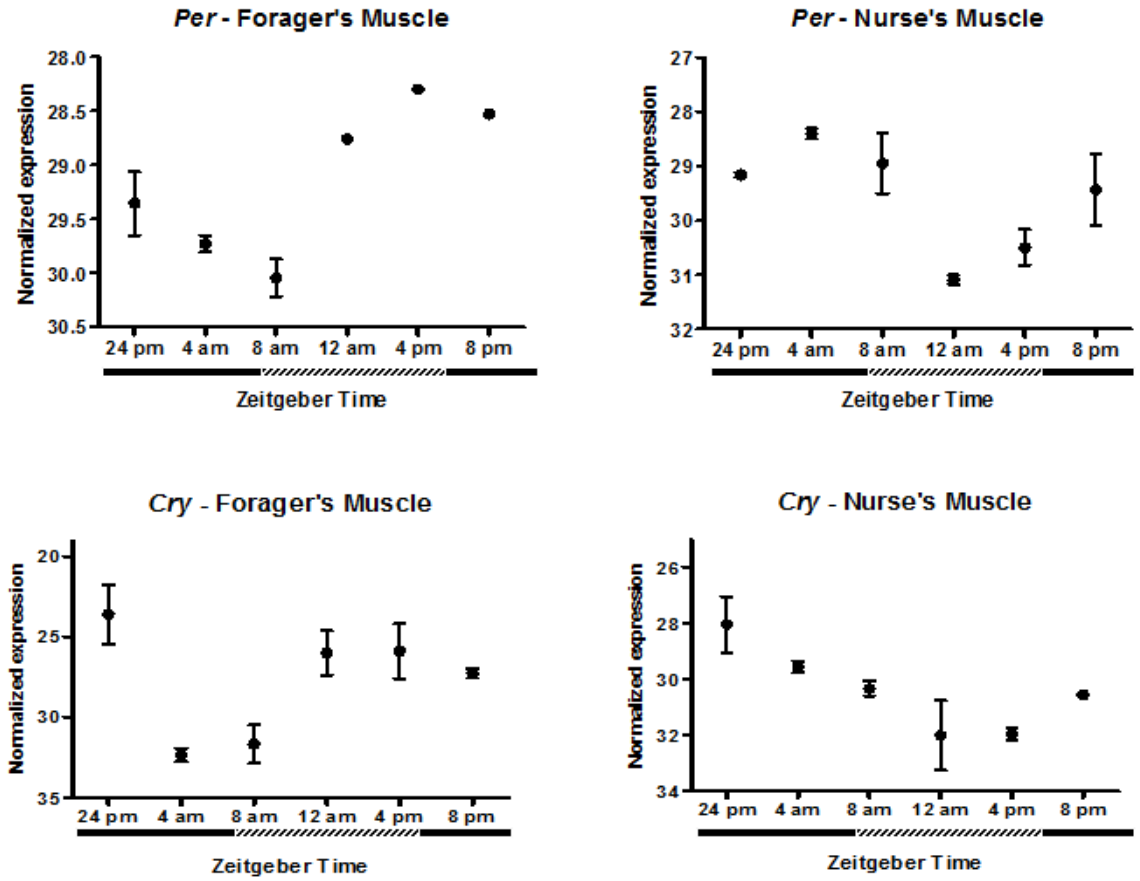


Fig.7. mRNA levels of core clock genes, relative to the internal control gene *amRPS5*, in the honey bees flight muscle tissue, over a 24-h period. Each time point represents the mean ± SEM for the experiment (n=6). Expression of *per* and *cry* demonstrated a significant variation over time ($P<0.05$, repeated measures ANOVA). The horizontal bars across the down of each graph represent the duration of the light (white bar) and dark (black bars) phases of the prevailing natural light-dark cycle.

Likewise, *Cry* was expressed in phase with *Per* showing a peak that was ~ 6.5 and ~ 4 – fold higher than its lowest respectively in foragers and nurses (4 h prior than the peak expression of *Per* genes, Tab.1). The presence of tissue specific daily oscillation of the core clock gene *Per* expression, especially in the case of nurses, rises the suggested idea that a peripheral clock in the flight muscle may exist and if it is so, such oscillator may be entrained by another external cue

than light, since bees didn't experienced any exposure to external photoperiod while they were inside the colony under total darkness.

2.3.2 Circadian gene expression patterns varies with age in brain and flight muscle

Assuming that *Per* is a critical component of the circadian clock (Rubin *et al.*, 2006) and its expression phase has been previously documented in honeybee brain (Toma *et al.*, 2000; Shimizu *et al.*, 2001; Bloch *et al.*, 2003; Shemesh *et al.*, 2010; Eban-Rothschild *et al.*, 2012), it was important to test whether *Per* expression profile, monitored each 4 h, differs or not from brain to muscle, and to which level in nurse compared to forager collected from the field colony maintained under natural environment. A striking shape similarity has been clearly detected in the cyclic expression of *Per* mRNA between brain and flight muscle tissue in old bees, as was found two peaks around ZT16 and ZT8 (One way ANOVA, $p < 0.05$ and $p = 0{,}0008$ respectively in forager's brain and flight muscle, Tab. 1) (Fig. 8). By contrast, their young sisters clearly showed a lack of synchrony in *Per* mRNA expression between tissues. *Per* transcript levels varied throughout the day in nurses only in flight muscle tissue, consisting of a peak occurring at the beginning of the photophase, whereas no significant differences in average daily levels of *Per* expression were observed at brain level, as reported in previous analyses of honey bees (Toma *et al.*, 2000; Bloch *et al.*, 2003), thus validating our experimental procedures. This in-hive experiment, showed how, in young nest workers that are contextually active around the clock, a flight muscle tissue in which a *period*-dependent peripheral clock tend to oscillate in an independent manner from the well known attenuated brain pacemaker cells (Shemesh *et al*., 2010).

2.3.3 Circadian Clock regulation of muscle's specific gene "*Nautilus*" and division of labor

Under environmental LD conditions, we further quantified temporal expression patterns of *Nau* gene in flight muscle tissue, to compare its expression profile to *Per* transcript level, between forager and nurse bees and to verify whether *nautilus* gene could be clock regulated. *Nautilus*, a *MyoD* (myogenic determination factor) family member, has been particularly selected according to its important role in the development of skeletal muscle in *Drosophila* embryos (Michelson *et al.*, 1990) but also for the classification of its mammalian homolog *MyoD 1,* as a clock controlled gene in rodent skeletal muscle (McCarthy *et al.*, 2007). Results from ANOVA, showed a strong and

significant 24 h rhythmic component for *Nau* for either nurse and forager bees (respectively p = 0.0003 and p = 0.0007) in flight muscle tissue level.

Table 1 - Peak times, trough times, and fold increases of genes found to significantly vary over time in the case of nurses and foragers bees, using one-way ANOVA

Gene Symbol	Peak (Clock Time)	Trough (Clock Time)	Fold Increase	*P* Value
Forager's brain:				
• *Per*	1600	0800	1.25	0,0044 (**)
• *Cry*	1200	0000	1.09	0,0383 (*)
Nurses's brain:				
• *Cry*	2200	1200	2.43	0.0105 (*)
Forager's flight muscle:				
• *Per*	1600	0800	1.75	0.0008 (***)
• *Cry*	1600	0400	6.47	0.0317 (*)
• *Nau*	1600	0800	5.20	0.0007 (***)
• *Pgm 2*	1600	0400	3.06	0.0399 (*)
• *Tmod*	2000	0400	8.78	0.0047 (**)
Nurse's Flight Muscle				
• *Per*	0400	1200	2.69	0.0355 (*)
• *Cry*	0000	1200	3.95	0.0295 (*)
• *Pgk*	1600	0400	2.9	0.0025 (**)
• *Nau*	0400	1200	4.34	0.0003 (***)
• *Pgm 2*	2000	0400	0.7	Ns
• *Tmod*	0400	1600	4.325	0.0028 (**)

(* (P<0.05); ** (P<0.01) and *** (P<0,001), repeated measures ANOVA).

It is noteworthy that the transcript level of *Nau* gene was found to be expressed in a coherent phase with daily *Per* brain and flight muscle mRNA level, displaying the significantly highest peak in the evening at ZT16 (Fig. 8). Although *Nau* transcript manifested phases and variations of amplitudes similar to those of the main core clock genes *Per* and *Cry* over the 24 h sampling period of time, such circadian regulation was only limited to the peripheral level in the youngest bees. In its circadian cycling, *Nau* mRNA exhibited maximal peak of expression at ZT4, exactly 12 h antiphase to the *Nau* peak in foragers. This could lend credence to the robustness of such peripheral clock oscillator, that succeed in maintaining its own 24 hour molecular rhythm and driving rhythmic expression of such specific muscle precursor *Nau* gene specifically when it is uncoupled from the locomotor activity controlling centers, according to an age related division of labor.

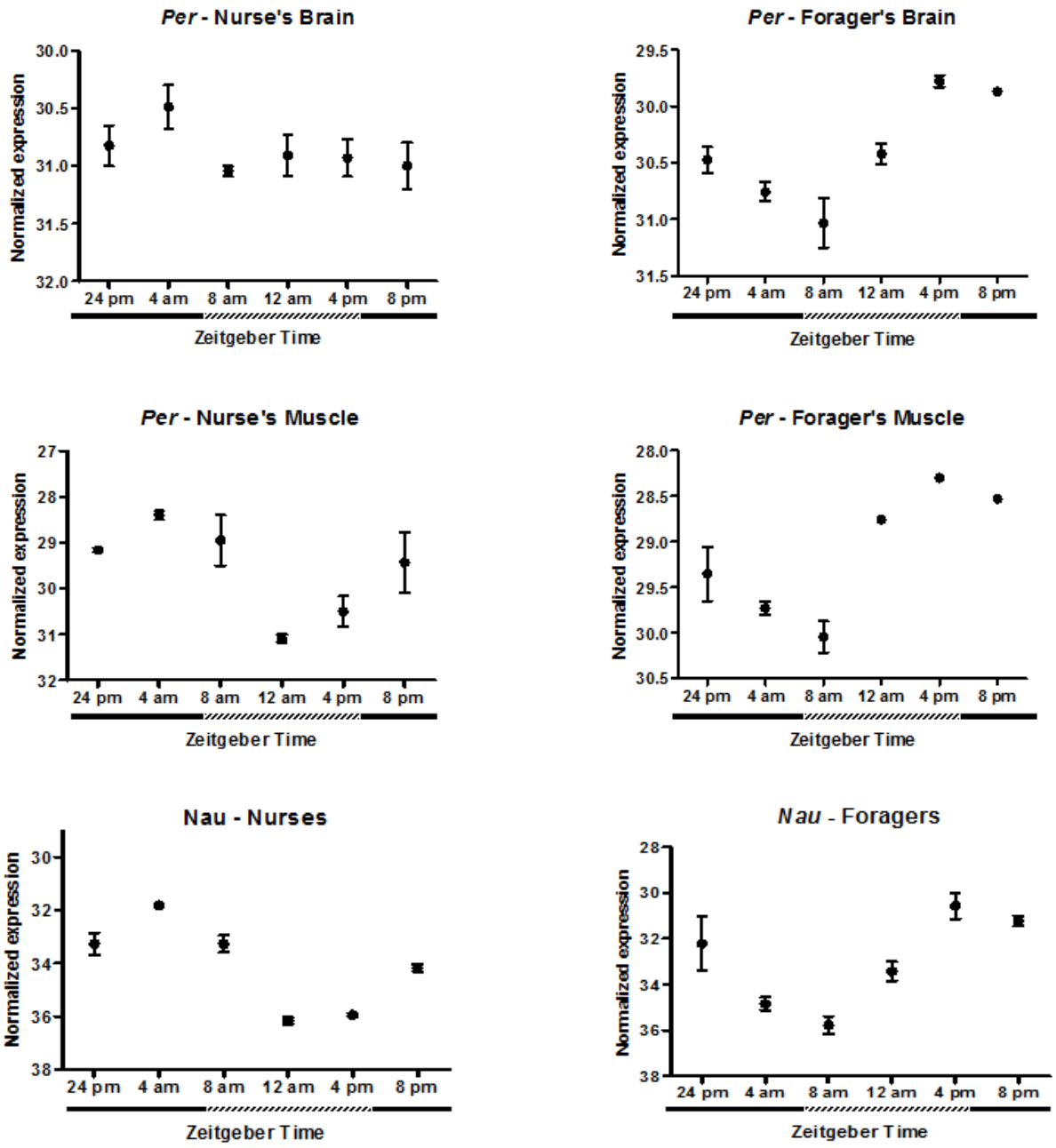


Fig.8. Age related differences in mRNA levels of core clock genes and clock controlled gene *nau*, relative to the internal control gene *amRPS5*, in either brain or peripheral flight muscle tissue of the honey bees, over a 24-h period. Each time point represents the mean ± SEM for the experiment (n=6). Expression of *per* and *cry* demonstrated a significant variation over time ($P<0.05$, repeated measures ANOVA). The horizontal bars across the down of each graph represent the duration of the light (white bar) and dark (black bars) phases of the prevailing natural light-dark cycle.

2.4 Discussion

The present study, realized on free – flying colonies, provides new insights into the ontogeny of core clock components regulation at the peripheral flight muscle tissue in honey bees. For the first time, results obtained through the present reserach established that the cells of the flight muscle organs express the core clock genes *Per*, the clock – related gene *Cry* as well as the known muscle specific gene *Nautilus,* with an age – dependent difference in average daily level's pathways under the 11:13 – h light – dark natural photoperiod regime. Anyway, since the approach we used was limited to field sampling of the behaviorally well defined rhythmic (foragers) and arrhythmic (nurses) type of worker bees, to basically identify any differences in daily transcript level in such peripheral tissue, our findings would give promising support to further researches, aiming to unveil the endogenous nature of any possible peripheral clock located in such part of the honey bees body.

2.4.1 Flight muscle tissue expresses robust circadian oscillations for circadian genes

Many are the studies focusing on the transition from nursing to foraging in honey bees. Impressive advances have been made in our understanding of the changes that rely to ontogeny of the endogenous circadian rhythmicity, but the mechanism that underlie the plasticity in behavioral rhythms and its relationship to the circadian system is still very poorly known and confined exclusively to the central coordination located in the brain. As workers tend, in an age related fashion, to switch from caring for brood around the clock to foraging with a robust diurnal rhythm, we considered of unprecedented value tuning our research to the circadian molecular pathways linked to task – related chronobiological plasticity in flight muscle machinery, for which neither *Per* molecular rhythms nor behavioral rhythms have been previously described in bees. Furthermore, our choice was reinforced by recent intriguing findings related to changes in main core clock gene's expression in honey bee brain, that were primarily driven by behavior than by age (Toma *et al.*, 2000; Bloch *et al.*, 2001, 2003, 2004; Shemesh *et al.*, 2007).

When testing the flight peripheral tissue in the current field part of the research, strong 24 h rhythms were found for each of the core clock genes selected in the case of nurse and forager bee's workers. However, if, as our results seem to indicate, such gene's temporal expression

profile, although were daily fluctuating in most type of workers, showed a consistent task – specific differences in oscillation patterns. In fact and under natural environmental context, *Per* and *Cry* mRNA peaked in the early evening in the case of foragers, in correspondence with locomotor activity patterns. In contrast, young nest workers showed a nocturnal cycle in clock genes temporal oscillations in flight muscle, respectfully to natural illumination regime, which add incongruities to earlier observations categorizing the youngsters dedicated completely to brood tasks as exclusively arrhythmic bees. This findings provide a consistent support to the hypothesis that the structured age related division of labor implies also the presence of functionally autonomous local circadian clocks that behave differently from the brain – central located oscillator and may be involved in coordinating many physiological processes in a tissue – specific fashion, that fits with life history tactic. At this level, the important question to ask is the following: *if a developmental chronobiological plasticity in bees is mediated by a peripheral reorganization in the circadian system, that integrate the coupling of central and peripheral clocks at an older age, which environmental zeitgeber is responsible of entraining the phase of flight muscle circadian rhythm of younger nurse?* Here, we will present two independent lines of evidence that, in combination, strongly suggest that nurse tasks could support the physiological role of the clock, that is hypothesized to oscillate autonomously in flight muscle at a specific time of honey bee life span. Inside the colony, workers with the specific task threshold (Jones *et al.*, 2004) succeed in keeping the brood nest temperature between 33 and 36 °C and optimally at 35° C (Seeley & Heinrich, 1981), for optimal brood and flight ability development (Harrison, 1986; Oskay, 2007) by fanning hot air out of the nest when temperature are highly elevated or clustering together to generate metabolic heat when it is highly cold; thus, the flight muscles can be promoted as source deeply involved in mechanisms of thermal homeostasis (Stabentheiner & Schmaranzer, 1987) and this independently of the kind of behavior expressed. During sampling period, the average ambient temperatures were fairly stable and close to 26,05°C, but varied by as much as 6 degrees throughout the day, being, during the late night – early morning, apparently lower than the ideal for nest temperatures; this is making us to suggest a probability of a daily mechanism for effective nest thermoregulation driven by various metabolic processes, whose entrainment and phase are dependent upon a local pacemaker. Such overt of endothermic activity has been also demonstrated by Engels *et al.* (1995) when ambient temperature was lower than that of the brood nest. A crucial point is that, according to such explanation, the ambient temperature cycle that directly entrain metabolic processes in nurses will also receive such responsiveness from the

foragers, which will express the same circadian phase patterns of clock genes expression than the previous. Nevertheless, our results showed exactly the inverse entrainment effects. This might be due to the fact that foragers, by spending more time outside the hive, are exposed differently to the environmental phase resetting cues than do nurses inside the dark and thermoregulated hive. Previous researches have reported a fundamental conjunction between the light and temperature entrainment pathways of the honey bee circadian clock system (Moore & Rankin, 1985; Fuchikawa & Shimizu, 2007), according to whether the overt behavior occurs in response to either cycle alone or combination of the two together (Moore & Rankin, 1993). In *Drosophila* (Boothroyd *et al.*, 2007), it is thought that effect of both light and temperature tunes a synergism that allows a full organism adaptation to the seasonal changes in the surrounding environment.

Alternatively, and since the daily in-hive temperature cycle is, in itself, a complex aspect of combination of external active regulatory processes and internal passive effects (Southwick, 1991; Jones *et al.*, 2004; Stabentheiner *et al.*, 2010), the individual changes in body temperature may also account itself for the entrainment of the individual nest mate flight muscle circadian rhythms, and consequent dynamics of colony thermoregulation. Temperature is well known as being one of the most robust physiological time –giver of circadian phase in the most of the organisms. In diurnal mammals for example, body temperature tend to exhibit a robust circadian profile generally with high peak – trough fold increase during early evening than in the morning (Refinetti & Menaker, 1992) relying this to the fact that diurnally active mammals can be considered to be in a “heat-gain” mode in the morning and a “heat- loss” made in the evening (Aldemir et al., 2000; Waterhouse et al., 2004; 2007) due to a circadian regulation of metabolic rate. In honeybee, a similar elevation in body temperature has been identified following 7 to 8°C elevation over the daily cycling of ambient temperature, in addition its rhythmicity continued to free-run with a 24 h period of time, even under constant darkness. To our knowledge, little is known about the effect of temperature on clockwork machinery in social synchronized honeybee insect, but results from our studies, could make tempting to support the idea that the persistence of the *period* mRNA cycling in the nurse’s *per*-flight muscle expressing tissue is more entrained through a kind of feedback loop existing between body temperature and metabolic activity rather than by the central pacemaker in the brain. As a consequence, questions are risen regarding the autonomy of such flight muscle clock, especially in the case of foragers, which temporal pattern of *period* mRNA followed similar phase of oscillation in both central and peripheral tissues. Recent research in

mammals have demonstrated that although circadian body temperature fluctuations are peculiarly dependent upon the central clock, which regulation occurs indirectly via control of sleep-wake cycles and activity patterns, has sustained circadian rhythmicity in a cell-autonomous fashion through a notable capacity to slow the damping of an existing rhythm while analyzed in vitro (Brown *et al.*, 2002) . Thus, in honey bee, it is likely possible that even if body temperature together with behavioral rhythms is independently controlled by a common biological oscillator (Fuchikawa &Shimizu, 2007), could serve as indirect route by which the brain entrains peripheral clocks under normal environmental conditions. Such suggestion requires further research to determine the mechanisms by which interaction between thermoregulation and core body temperature, is able to maintain persistent temporal expression patterns of clock genes in honey bee flight muscle tissue, according to their division of labor.

2.4.2 Circadian Clock regulation of muscle's specific gene "*Nautilus*" and division of labor

As a mean to gain insight into the existence of a circadian peripheral clock in honeybee flight muscle tissue, we further moved to investigate the oscillation pattern of *nautilus* expression, in either nurse or forager worker's *per*-flight muscle expressing tissue. As previously cited, *nautilus* is a *MyoD* (myogenic determination factor) family member, that has been particularly selected according to its important role in the development of skeletal muscle in various vertebrate and invertebrate organisms (Michelson *et al*., 1990; Tapscott, 2005; Andrews *et al.*, 2010) but also for the classification of its mammalian homolog *MyoD 1*, as a clock controlled gene in rodent skeletal muscle (McCarthy *et al.*, 2007). *Nautilus* satisfied our criteria for circadian transcripts, as *nau* mRNA was expressed in a circadian fashion in honeybee's flight muscle. Although its phase followed a striking differences according to division of labor, the detection of myogenic regulatory family member, as constitutively expressed in phase with *period* in the worker's flight muscle tissue, is an interesting finding that supports previous reports underlining the important role played by the mammalian homolog *MyoD1* gene in the daily regulation and maintenance of muscle tissue (Martin *et al.,* 2010). Taken into account the last considerations, it is likely relevant to suggest *nautilus* as a clock controlled gene (CCG), whose temporal expression is regulated directly by the flight muscle molecular clock. Thus, such regulation is tightly associated to the cellular clock contribution in the daily maintenance of muscle phenotype and function.

3. LABORATORY RESEARCH

3.1. Introduction

In almost all organisms, the circadian rhythms constitute relevant biological rhythms that respond to the following three fundamental characters: 1) they persist or free run with a period closer to 24 h, under constant conditions; 2) they can be entrained by external factor cues or "zeitgebers", mainly represented by daily fluctuations of light and temperature; 3) they are temperature compensated. The core clock regulating the circadian timing of many physiological and behavioral functions is composed of a cell autonomous - *pacemakers*, which molecular mechanism consists of a number of clock genes (CGs) and their resultant proteins that form an interlocking transcription-translation feedback loops with positive and negative elements. The clock cells of animals are interconnected in a well defined network that effectively coordinates their activities and orchestrates their overt rhythms, so that biological processes may opportunely occur in synergy for organism fitness (reviewed in Allada & Chung, 2010).

In mammals, it is now well established how the feedback loops regulate the expression of a set of genes outside the core clock, called core clock controlled genes (CCGs), that can be expressed in several cell types (Akhtar *et al.*, 2002; Panda *et al.*, 2002; Storch *et al.*, 2002), peculiarly in skeletal muscles (Zambon *et al.*, 2003; McCarthy *et al.*, 2007; Martin *et al.*, 2010). Recent evidences suggested that additionally to the core clock genes, 8-10% of the transcriptome is regulated in a circadian fashion, with specific gene's expression between one type cell to another (Akhtar *et al.*, 2002; Panda *et al.*, 2002; Zambon *et al.*, 2003) in response to the tissue's need when changes in the external environment occur. Moreover, the muscle transcriptome that was found to include components of the mammals molecular core clock, named as CCGs and known to be under a coordinated daily regulation, could highlight a down-regulation of a set of known genes with important muscle specific functions, such as *actin* and *titin* in case of clock mutant mouse (McCarthy *et al.*, 2007). Indeed, under the light of such findings, basis have been provided for better understanding the role of circadian rhythms in the daily maintenance of skeletal muscle (McCarthy *et al.*, 2007). Regarding to the significant circadian regulation of human (Kline *et al.*, 2007) or equine (Martin *et al.*, 2010) athletic performance, it was suggested that the robust cycling of muscle transcriptome in both these animal species may contribute considerably to overt

rhythm in their performance or additionally to the generated 24h rhytmicity in cardiorespiratory factors (Millar-Craig *et al.*, 1978; Giacomoni *et al.*, 1999; Spengler *et al.*, 2000; Piccione *et al.*, 2009; Martin *et al.*, 2010). This observation is especially important and, in addition to recent findings reporting the effectiveness of the time of training as a circadian phase resetting agent in humans (Edgar & Dement, 1991; Zambon *et al.*, 2003; Atkinson *et al.*, 2007), it has been of great contribution in suggesting the most advantageous time of the day for training and maximally performing, thus avoiding exposition of organism to disruptions or damage such as musculoskeletal injury and fatigue. To date, no evidences for a potential circadian variation in muscle capacity and subsequent adaptations to the external environment has been conducted in insects, in contrast to mammals studies.

In its broadest sense, the synchrony of honey bee individual as with both its external and internal environments is critical to the colony Superorganism's well-being and survival. For example, the adult honey bees spend the first 2 to 3 weeks inside the hive, taking care of brood around the clock, without any rhythm overall their behavioral activity (Bloch, 2009, 2010). Later, they switch naturally to foraging activity exhibiting a strong circadian rhythm, so they are active during the day and rest by night, according to their dependence to the diurnal food source availability (cited in Rodriguez-Zas *et al.*, 2012). Although the division of labor in honey bees generally relates to age, the natural plasticity in circadian rhythm to which it is associated is not absolute; in fact, we may say that it is rather context dependent. For example, moving the nurses from the hive to individual cages under constant conditions, where they are maintained in isolation and without any contact with conspecifics, triggers a robust circadian rhythm overall their locomotor activity and clock genes expression (Shemesh *et al.*, 2007, 2010). Such observations led to repudiate the clock arrhythmicity of young bees as a theory, and are recently making attempts to suggest the presence of other oscillators, which keep on measuring time within the circadian system of arrhythmic young bees (reviewed in Bloch *et al.*, 2013). In light of the growing association between the presence of clock cells outside the central oscillator and the molecular reorganization of the clockwork according to the context environment, it will also be important to understand the potential of such synchronized system in driving rhythmic expression of clock controlled genes involved in physiological and metabolic functions, at the peripheral level.

Recently, initial research based on gene profiling studies, reported that the known core clock genes have been found to show a circadian pattern of expression in the honey bee skeletal muscle

(see chapter 2). Interestingly, by comparing the gene oscillating expression between foragers and nurses collected from the field, it was intriguing to note that, if a circadian clock exists in the flight muscle, the degree of synchrony that coordinate such peripheral oscillator to the central one, located in the brain, differs highly according to the age of worker bees. There is precedent for differences in the organization of the circadian system that is associated with the division of labor. Microarray gene expression experiments identified a number of oscillating transcripts, involved in a wide range of biological processes and molecular functions, that have shown circadian pattern of oscillations in active around the clock nurse bees rather than in the foragers (Rodriguez-Zas *et al.*, 2012). In a few words, this findings have highlighted that the mechanisms that were able to couple the central and peripheral oscillators in the case of foragers, may be somewhat different from those that unlikely led to the uncoupling in nurses. In social insects, the identity of these mechanisms and the evidence of social context influence on their downstream action on peripheral tissue, are yet to be determined. It is noteworthy, that identifying the nature of the *per* flight muscle oscillator must be a good start for these sort of studies.

To our knowledge, results from the field studies reported in chapter 2, although revealed the presence of circadian oscillator genes in flight muscle of honey bee workers, could not be sufficient to support the endogenous nature of such clock, since it may be possible that the rhythmicity in gene expression was influenced by other factors (such as social ones, like the presence of brood or foragers), which are independent from an endogenous circadian regulation. Indeed, a rhythm is defined as circadian if it persist in absence of environmental cues, with a period of or closer to 24h, under constant conditions. Therefore, considering all have been said above, a second and indispensable part of the research, mainly to be conducted under laboratory conditions, has been planned with the following specific aims :

1) Use the real-time reverse transcription polymerase chain reaction (RT-PCR) assays and a robust normalization strategy, investigating the circadian patterns of core clock genes expression in the honeybee flight muscle, in relation to the subjective light-dark cycle in honeybees exposed to different cycle of continuous darkness, at different key ages. The resulting detailed expression could provide evidences for an ontogenetic basis for circadian coordination of molecular mechanisms that undergo flight machinery during behavioral development in the bees, across their life span.

2) Verify the hypothesis that the expression of selected responsive genes, highly involved in the metabolism and physiology of the honeybee's muscle, is under control of the circadian clock mechanism.

3) Compare the clock gene expression profile between brain and flight muscle tissue at each age sampling.

This laboratory part of the research has been thought with the aim to try providing functional insights related to the nature of the coordination existing between oscillators, in association with the developmental related circadian regulation of the flight muscle machinery in honeybee.

3.2 Materials and methods

The whole laboratory part of the research presented in this report has been realized at the University of Catania (Italy), with honey bee material coming from the apiaries of the Agricultural Experimental Farm of the campus and most of the laboratory work realized thanks to the facilities of the Department of Agri-food and Environmental Systems Management (DiGeSA), especially the Biotechnology Laboratory, the Agricultural Zoology Laboratory and the Climatic Chambers and Incubators Unit. A small part of the laboratory activity presented in the following pages has been kindly hosted by the Molecular Biology Laboratory of the Department of Agricultural and Food Science (DISPA).

3.2.1 Experimental design

3.2.1.1 Housing colonies

Colonies of European honey bee (*Apis mellifera* L.) (with a population of about 20,000 bees each), coming from the apiaries of the Agricultural Experimental Farm of the University of Catania and used as source of material for the laboratory research, were maintained during the whole experimental activity in the local courtyard facility at the Department of Agri-food and Environmental Systems Management of the University of Catania. The main part of the samplings planned for the experiment were conducted at the time of year (mid January – mid February)

corresponding to an approximate 10 h light/14 h dark (LD: 10:14) natural photoperiod (sunrise at 7:00 AM and sunset at 4:50 PM, on average) and ambient temperature varying closely from maximum =16°C and minimum =5°C.

Before starting the samplings, care was taken in order to control the exposure of bees to extrinsic factors, which could had influence on the later functioning of their biological rhythms. Thus, the bee hives were confined in an "environmental chamber", made of an opaque fabric 2x2x2 m (Fig. 9). By this way, exposure of foragers to light was limited to day, when bees fly outside to forage, and to complete darkness during the night, when the hive was hidden from all possible sources of external light; by contrast, nurses were mostly still experiencing constant dark environment, inside the hive. Bees were fed *ad libitum*, from their natural food which was occurring directly inside their colony. It is noteworthy that all manipulations and collections that have been made during the experiment, have been realized under dim red light, which is considered as not distinguished by the bees (Von Frisch, 1967).

Fig. 9. "Environmental chamber" for housing the hive.

3.2.1.2 Collection of samples

In order to obtain the 1 day old individuals, brood combs showing a relevant presence of mature pupal cells have been taken from the source colonies and placed inside an opaque Plexiglas box (70 cm W x 55 cm L x 60 cm H), under constant conditions of darkness (DD), inside an incubator

(35°C ±0,5 °C of temperature; Relative Humidity = ~ 50%) till emergency of adults. During the first 2 days after introduction of the combs, each adult emerging was paint marked on the thorax, with 3 different colors, respectively corresponding to 0-24h; 24-36h and 36-48h after the isolation of the combs. By this way, all adults emerged from the combs were subdivided in emergence-age-homogeneous groups, so that their age could have been inferred when they have been collected.

Aiming to test the circadian regulation patterns of honey bee tissue while removed from the hive environment and across their life span, we transferred each marked 1-day old bee to individual perforated eppendorf tubes (d=2 cm; length=20cm), which were supplied by water and candy food in each cap at their extremities. This guaranteed an *ad libitum* feeding way for bees during their isolation and also eliminated any need to provide each individual by water and food daily. The tubes were maintained successively fixed into a frame (Fig. 10).

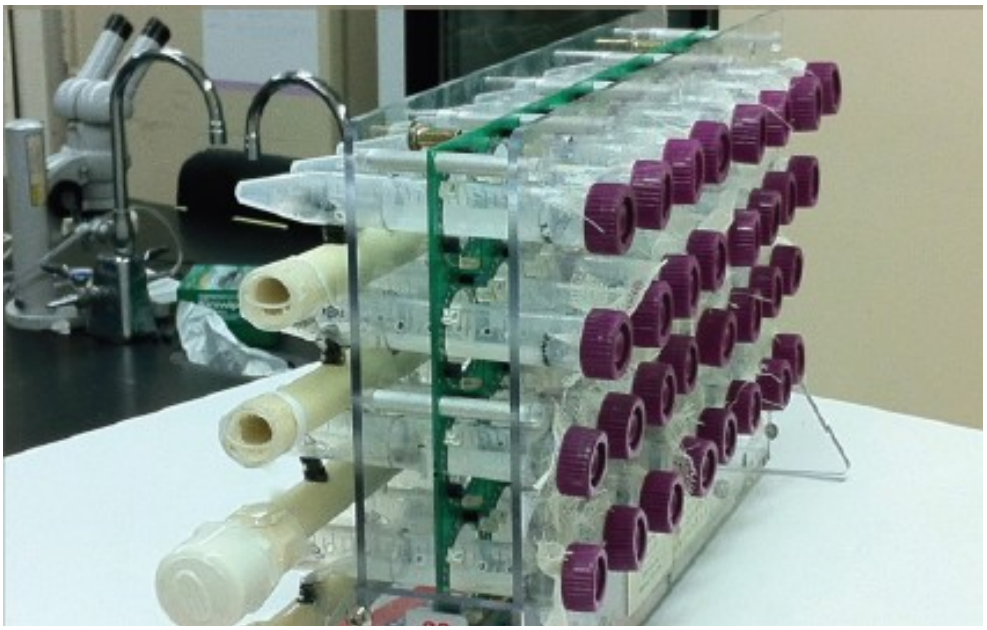


Fig. 10 - Individual Eppendorf tubes into the frame to place individually bees under constant conditions of darkness .

The collection of honeybees for mRNA analysis has been realized at 5 different days of age (1, 3, 7, 17 and 24 days old), opportunely selected as they are considered to be key steps through the temporal division of labor that honeybees tend to exhibit across their life span. The gene expression profiles resulting from sampling realized at each age will provide a screening related to the ontogenesis of the molecular circadian regulation according to the age-related division of labor, in association with the development of honeybees. During the whole experiment, each

sample (N= about 8 bees/time point/age old) was collected at 6 different time points during 24h, as already seen in chapter 2.2.1. The frequency (every 4h) and duration (24h) of sample collection was required to establish the 24-hr repeating oscillation pattern of circadian mRNA expression. Bees for analysis were directly introduced in the liquid nitrogen and stored after at -80°C until brain and flight muscle dissection.

3.2.2 Brain and flight muscle dissection

After storage, brain and flight muscle tissues were isolated and dissected as previously seen in chapter 2.2.2, keeping frozen the dissected organs in dry ice during the entire dissection. As for the field research, all intact brains and muscles were stored individually at -80°C until mRNA analysis.

3.2.3 RNA isolation

Total RNA (Ribonucleic acid) was extracted (at each time point and age) from 2 replicated pools of 4 brains each (total of 60 brain samples) and 2 others of 4 muscles each (total of 60 thoracic muscle samples), using the RNeasy Micro Kit (Quiagen, Valencia, CA) by following the manufacturer's instructions. RNA yields were cleaned using the DNAse treatment (Qiagen), in order to remove any contaminating DNA. The concentration and purity of RNA was determined using a UV spectrophotometer (NanoDrop ND-1000) and the Agilent 2100 Bioanalyzer.

3.2.4 cDNA conversion

For each sample, 500 ng of total RNA was converted to cDNA (Complementary deoxyribonucleic acid) using high-capacity cDNA Reverse Transcription kits (Applied Biosystems). Following the manufacturer's recommendations, a reaction volume of 20 µl was used for each sample conversion. This 20 µl consisted of 2 µl of Reaction Buffer, 0.8 µl of 25×dNTP Mix (100 nM), 2 µl of RT Random Primers, 1 µl of Multiscribe Reverse Transcriptase, and respectful µl volumes of both molecular-grade water (Sigma) and of DNA-free RNA (500 ng of each sample). The resulting cDNA was stored at -20°C till qRT-PCR analysis.

To perform gene-expression testing, 1 µl and 2 µl were respectively removed from each brain and muscle sample, and then pooled. Dilution of the pooled samples with molecular grade water yielded a set of standards that were used to perform standard curves for each gene. The

remaining cDNA of each sample's reaction volume was diluted with a specific volume of water, in order to provide a working cDNA concentration.

3.2.5 Selection of genes and primers design

From the literature, in addition to the main core clock genes (*per, cry, tim, clock and cycle*), a panel of other genes potentially involved in the muscle metabolism (*GapDH, pgk, tmod, tpnt, pgm1, pgm2*) have been selected for gene expression analysis. Known sequences from Ensembl (Genome Browser) and NCBI were used to design primers for the selected genes, using Primer 3 software (Applied Biosystems) (Rozen & Skaletskyand, 2000), ensuring the specificity of the sequence by BLAST (Basic Local Alignment Search Tool) (www.blast.ncbi.nlm.nih.gov), preventing self complementarities and self-dimers with oligocalc and IDT websites.

Table 2 – Core Clock and Metabolic gene's Primers for Real-time quantitative PCR reactions carried out in this study.

Gene	Oligo	Sequence
List of Main Core Clock Genes (Source: Rubin *et al.,* 2006)		
amTimeless (Tim2)	Forward primer	AAATCGTCGTGGGAAACGGAGTCT
	Reverse primer	TTCTTCGTTCCCTTGTTCCTCGGT
amCryptochrome (Cry2)	Forward primer	AAGCTGTACCGCCACTTTCATTGC
	Reverse primer	CCCGTTTGACCGTTTGCCCATTTA
amClock (Clock)	Forward primer	GGATATTTTCGGTCTGATGTCGATAC
	Reverse primer	ACGTCCTGTACCCACGAAAAC
amCycle (Cyc)	Forward primer	TCAGGATGGCTGTCCAACACTTGA
	Reverse primer	AACCCTCCGCAGCTTGAAGTATGA
amEF1α	Forward primer	AGCAGTTGATCGTTGGAGTGAA
	Reverse primer	CATCCGGAGATCGGTACGAA
amRPS5	Forward primer	AATTATTTGGTCGCTGGAATTG
	Reverse primer	TAACGTCCAGCAGAATGTGGTA
amPeriod (Per)	Forward primer	AGCAATGACGAGGGTGGAAA
	Reverse primer	CTCTCTACTTGCCGAACTTGTTTG

List of Main Metabolic Genes		
am tropomodulin (tmod)	Forward primer	TTCGTGCAGAACTTCGTCGATCCT
	Reverse primer	ACGTAAATGTCACGGGAGAGGGTT
am troponin T (TpnT)	Forward primer	AGGCAATGATGCAGGCGATGAAAG
	Reverse primer	TTTCGTCTTGTTGCGCTCCAGTTG
amNautilus (nau)	Forward primer	CCAAGAAGCGCCGTTTCATTCGAT
	Reverse primer	ACTTCCTTCCACTTCCTTCCTGCT
am phosphoglycerate kinase (Pgk)	Forward primer	ACAGCTACTTGCGCTGCTAAATGG
	Reverse primer	TAATTCCAAACTTGCACCACCGCC
am phosphoglucomutase 1 (Pgm1)	Forward primer	AGATCTATGCCAACAGGTGCTGCT
	Reverse primer	TGCAAGACAAGCCCATATTCCGTC
am phosphoglucomutase 2 (Pgm2)	Forward primer	ACCTGATCCGGAATTCCCAACTGT
	Reverse primer	GCACAAGCTACTCTATCTGCATCTGG
am glyceraldehyde 3-phosphate dehydrogenase (Gapdh)	Forward primer	ACCGCTTTCTGCCCTTCAAATGAC
	Reverse primer	TTAATGGCAACAACCTGAGCACCG

3.2.6 Quantitative Real-time RT-PCR

Quantitative real time polymerase chain reaction (qRT-PCR) reactions were performed in an iCycler IQ Real time PCR Detection system (ABI 7500 instrument) and a master mix was prepared using 10 µl Sybergreen (Fast SYBR® Green Master Mix; lot N: 0901012), 3 µl of each primer, 5 µl of H_2O and 2 µl cDNA from each sample at each time point (duplicate/time point/bee) in 20 µl reaction volumes. Each qRT-PCR plate contained a standard curve, a negative control (NTC - did not contain cDNA in order to control for RNA and genomic DNA contamination) and the 20 µl combination of mastermix and cDNA each sample. Each well of the plate was produced in duplicate. The following experimental run protocol was used: quantification program with a profile of 90° C for 20 min, 95° C for 30 sec, and 60° C for 30 min, with a continuous fluorescence measurement. Target gene mRNA levels were measured by determining the cycle number at which amplification detection threshold was reached (C_T). The results were exported from the iCycler IQ Real-Time PCR Detection System into Microsoft Excel files for further analysis.

In the present study, the expression stability and level of candidate reference genes included in the brain and muscle tissues were assessed by qRT-PCR and data analyzed using the separate

reference gene stability analysis software package: GeNorm (Vandesompele *et al.*, 2002). "GeNorm, generates an M value for each gene which is arbitrarily suggested to be lower than 1.5 (with lower value indicating increased gene stability across samples) and a pairwise stability measure to determine the benefit of adding extra reference genes for the normalization process (again with a lower value indicating greater stability of the normalization factor). An arbitrary cut off value of 0.15 indicates acceptable stability of the reference gene combination" (Vandesompele *et al.*, 2002). For our samples, *RPS5* was found to be the most stably-expressed gene, with an M value of 0.393.

3.2.7 Data analysis

Daily variation of mRNA expression was statistically analyzed using repeated measures analysis of variance (ANOVA) with Graph Pad Prism software Version 4.0 for Windows (Graph Pad software, San Diego, California, USA, http://www.graphpad.com). The values of the relative expression of mRNA are presented as the mean ± SEM. A value of $P<0.05$ was considered significant.

3.3. Results

In this study, we compared age-matched honeybees from 5 different ages, individually isolated under constant conditions of darkness (DD) in the laboratory, to examine the gene expression across 24 h period of time associated with oscillator control in brain and flight muscle tissues across honeybee life span. For this goal, the values for the relative levels of each main core clock genes mRNA, either in brain or flight muscle tissue, was determined through qRT-PCR. The mRNA levels of the several genes involved in the flight muscle metabolism were measured only in the flight muscle tissue. The values of the relative levels of gene mRNA from each biological replicate (n=2) used, obtained at each of the 6 time points with 4 h intervals over the 24 h period of time, were averaged and the relative resulting oscillations abundance are represented in the following figures (Fig. 11 to Fig. 25).

3.3.1. Daily variation in the expression of clock genes in brain and flight muscle tissue according to age development of honeybee

The repeated measures ANOVA (n=6) revealed a significant variation in expression levels over time for the four core clock genes *per, cry, clock,* and *tim* (Figs 1, 2, 4, 5, 7, 8, 10, 11, 13 and 14). Interestingly, the circadian waveform features of the core clock gene were significantly variable from one age to another and especially in trend of synchronization between tissues analyzed. Moreover, a striking 24h rhythmic component was found for all four CGs in brain and flight muscle either in earlier (1 and 3 days old ages) and later aged honeybees (17 and 24 days old ages), although a circadian pattern has been significantly followed by all core clock genes in both brain and muscle tissues at 1 day old bees (see tab. 3), only by *cycle* gene in muscle tissue at 3 days old bees (see tab. 3), *cry* gene either in the muscle at 17 days old bees (see tab. 3) or in brain at 24 days old (see tab. 3).

Table 3 - Peak times, trough times, and fold increases of genes found to significantly vary over time in honeybee tissues at five different ages old, using one-way ANOVA

Gene Symbol	Peak (Clock Time)	Trough (Clock Time)	*P* Value
1Day old bee's brain:			
Per	0400	1200	0,0357 (*)
Cry	0400	1200	0,0066 (**)
Clock	1200	1600	0,0023 (**)
1Day old bee's flight muscle:			
Per	0400	1200	0,0008 (***)
Cry	0400	1200	0.0484 (*)
Clock	0400	1200	0,0299 (*)
Cycle	0400	1200	0.0299 (*)
tim	0400	1200	0,0037 (**)
pgk	0400	1200	0,0002 (***)
tmod	0400	1200	0.0225 (*)
3Days old bee's brain:			
Cycle	0000	0800	0.0288 (*)
3Days old bee's flight muscle:			
Cycle	1600	0400	0.0148 (*)
7Days old bee's flight muscle:			
cry	0400	1200	0,0227 (*)
pgk	0800	1600	0,0218 (*)
17Days old bee's flight muscle:			
cry	0400	1600	0.0223 (*)
24Days old bee's brain:			
cry	0400	1200	0.0011(**)
Clock	0800	1600	0.035 (*)
24Days old bee's flight muscle:			
pgk	1200	2000	0.0041 (**)

(* (P<0.05); ** (P<0.01) and *** (P<0,001), repeated measures ANOVA).

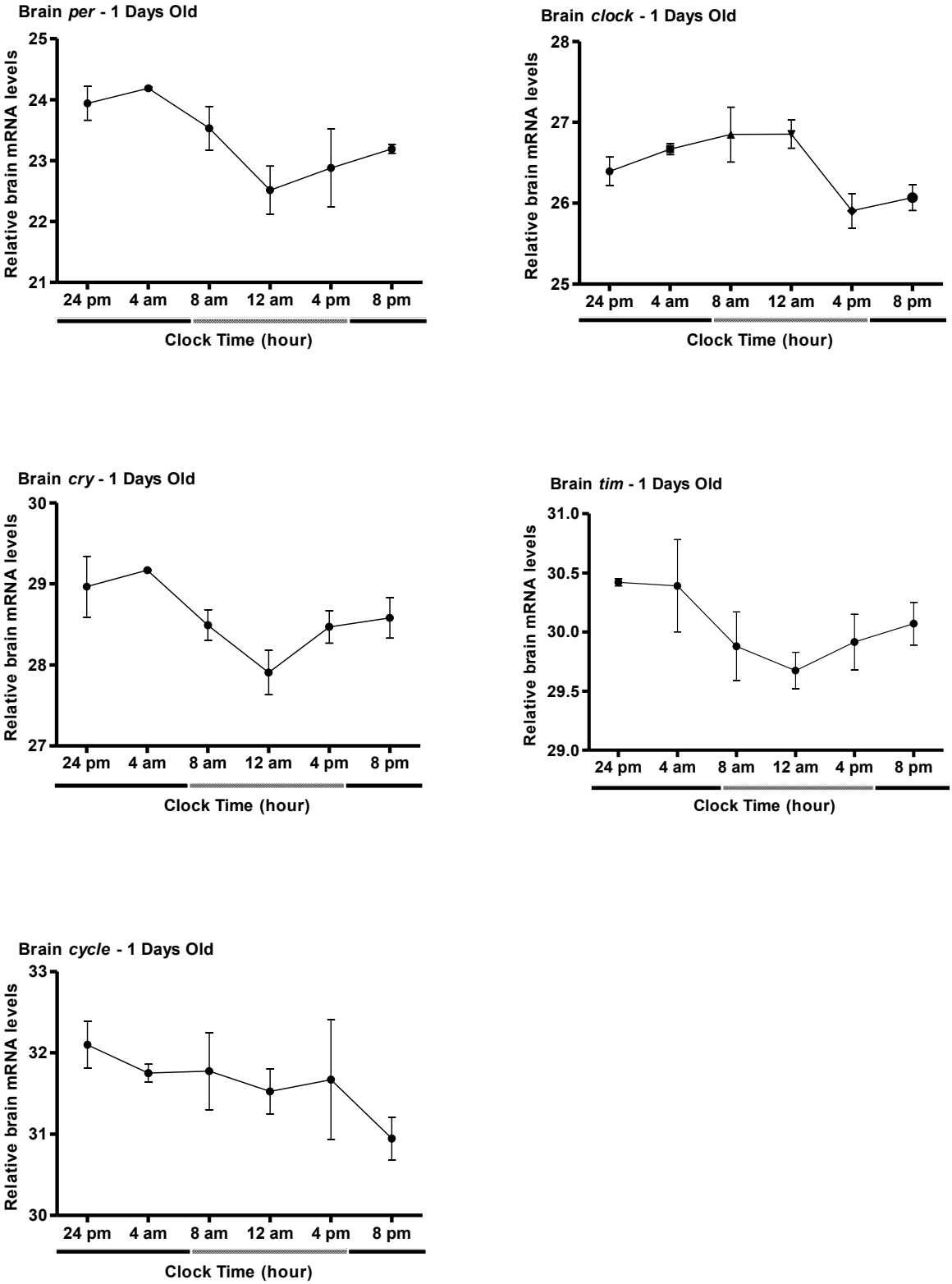


Fig.11. Twenty–four–hour profiles of 1-day-old honeybee brain core clock genes expression. Plotted are mRNA levels of candidate genes relative to the internal control gene *amRPS5*, in the honey bee brain tissue, over a 24-h period in constant darkness. Each time point represents the mean ± SEM for the experiment (n=8). The environmental dark-light cycle before isolation into constant darkness (DD) is depicted above each graph with the dark gray shading representing the subjective night and the light gray shading representing subjective day.

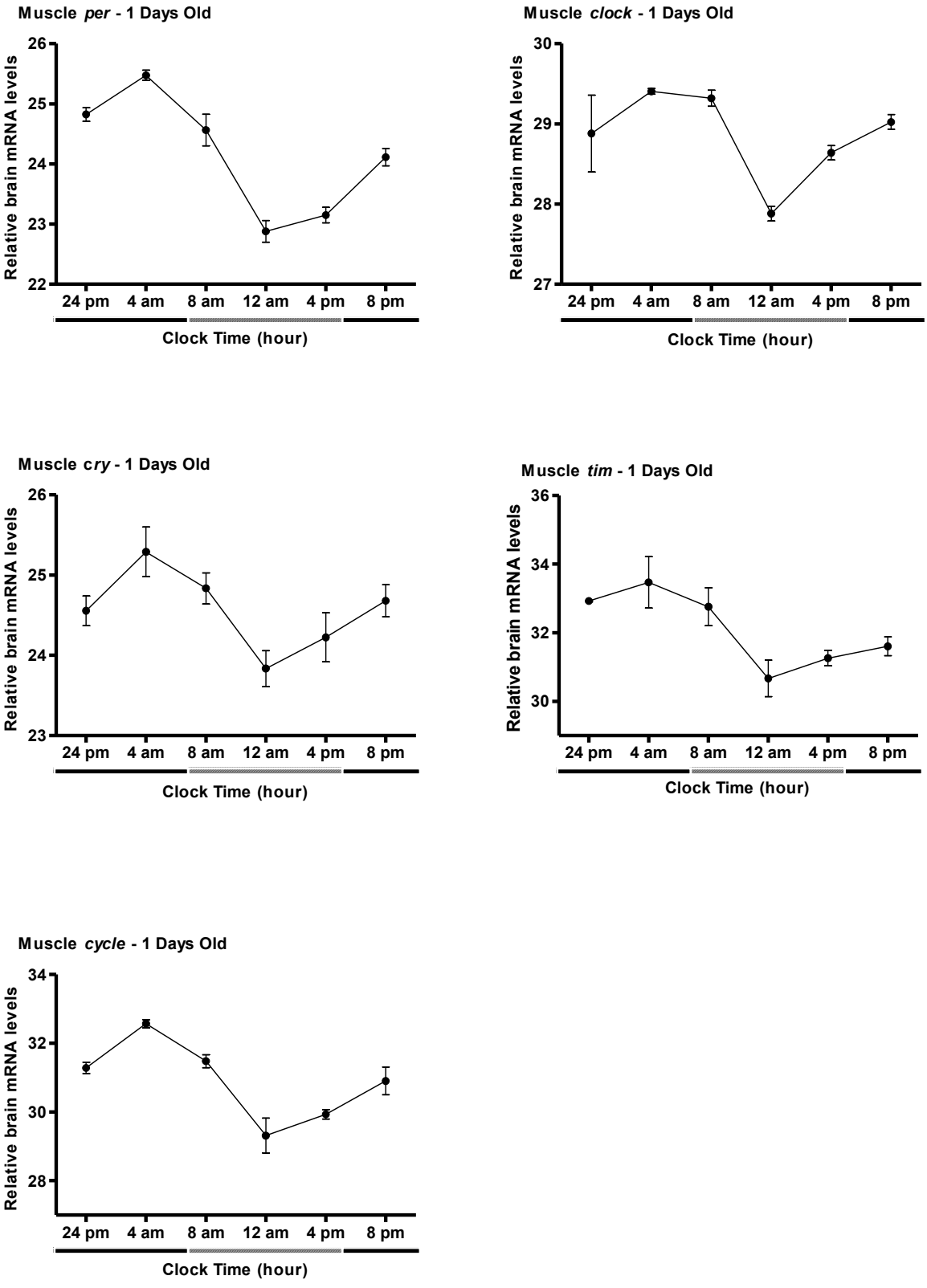


Fig.12. Twenty–four–hour profiles of 1-day-old honeybee flight muscle core clock genes expression. Plotted are mRNA levels of candidate genes relative to the internal control gene *amRPS5*, in the honey bee flight muscle tissue, over a 24-h period in constant darkness. Each time point represents the mean ± SEM for the experiment (n=8). The environmental dark-light cycle before isolation into constant darkness (DD) is depicted above each graph with the dark gray shading representing the subjective night and the light gray shading representing subjective day.

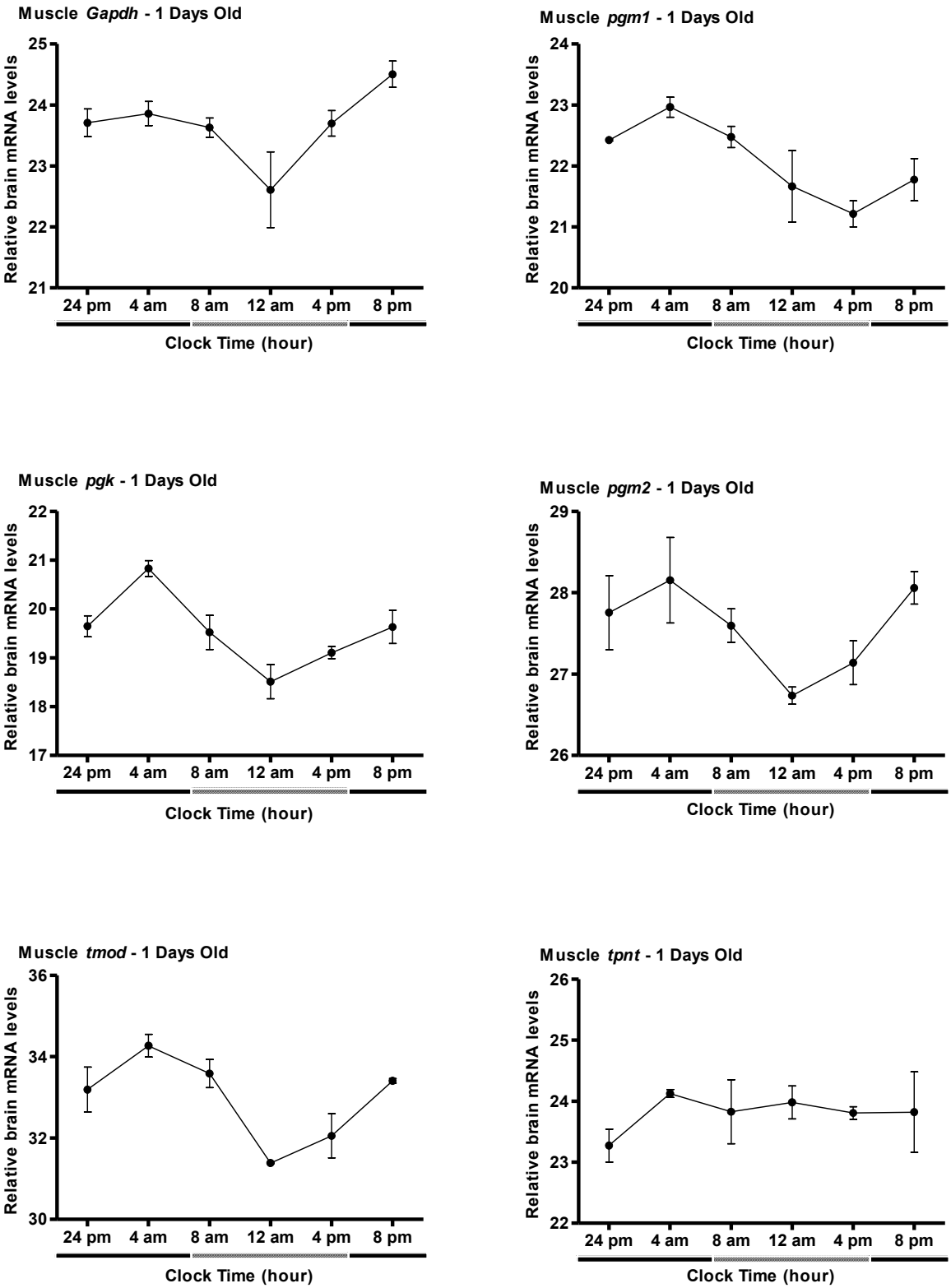


Fig.13. Twenty–four–hour profiles of 1-day-old honeybee flight muscle metabolic genes expression. Plotted are mRNA levels of candidate genes relative to the internal control gene *amRPS5*, in the honey bee flight muscle tissue, over a 24-h period in constant darkness. Each time point represents the mean ± SEM for the experiment (n=8). The environmental dark-light cycle before isolation into constant darkness (DD) is depicted above each graph with the dark gray shading representing the subjective night and the light gray shading representing subjective day.

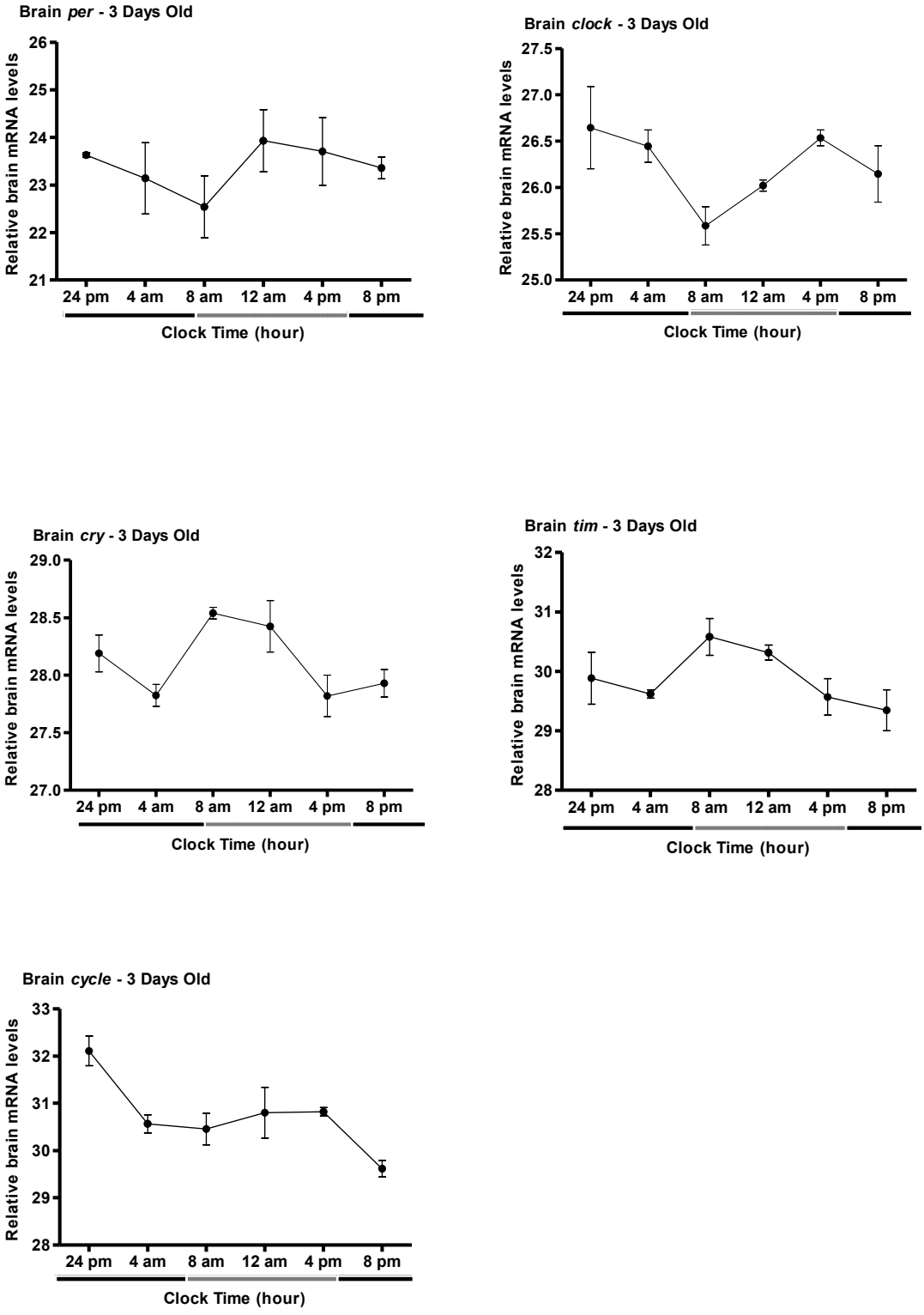
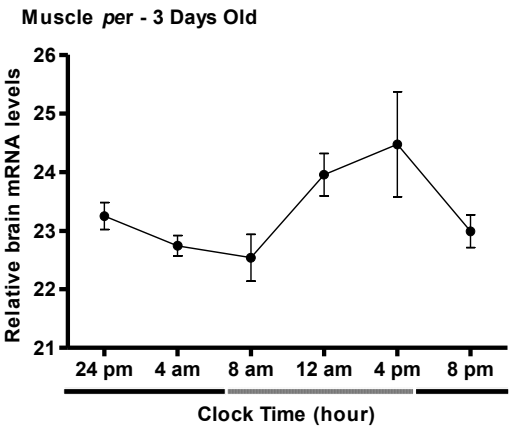
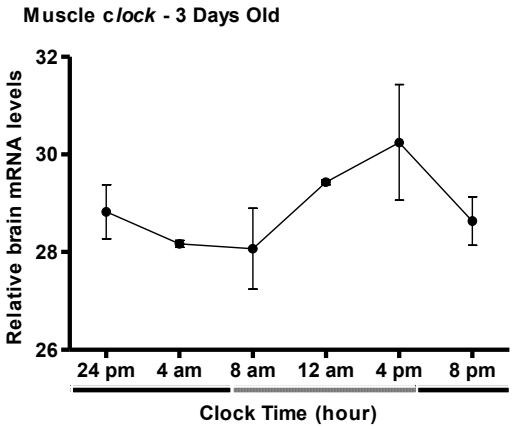
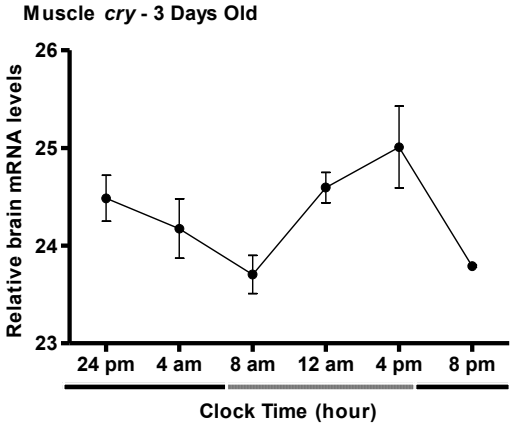
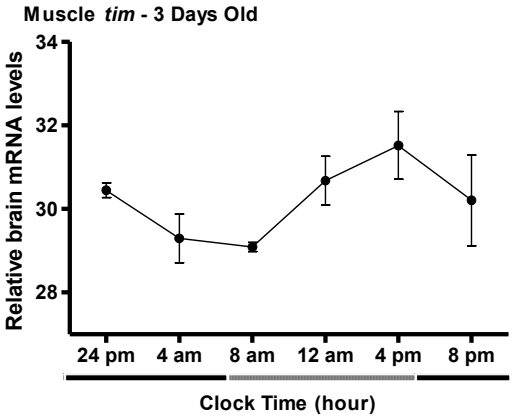


Fig.14. Twenty–four–hour profiles of 3-days-old honeybee brain core clock genes expression. Plotted are mRNA levels of candidate genes relative to the internal control gene *amRPS5*, in the honey bee brain tissue, over a 24-h period in constant darkness. Each time point represents the mean ± SEM for the experiment (n=8). The environmental dark-light cycle before isolation into constant darkness (DD) is depicted above each graph with the dark gray shading representing the subjective night and the light gray shading representing subjective day.









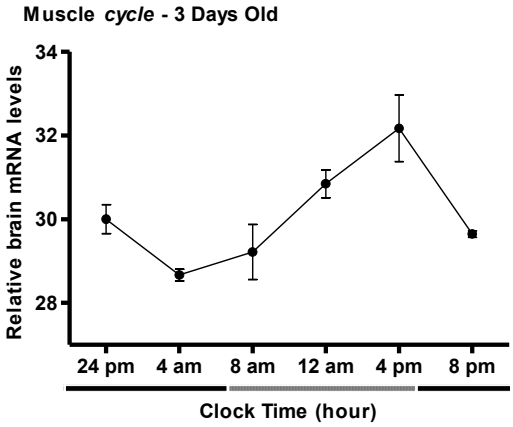


Fig.15. Twenty–four–hour profiles of 3-days-old honeybee flight muscle core clock genes expression. Plotted are mRNA levels of candidate genes relative to the internal control gene *amRPS5*, in the honey bee flight muscle tissue, over a 24-h period in constant darkness. Each time point represents the mean ± SEM for the experiment (n=8). The environmental dark-light cycle before isolation into constant darkness (DD) is depicted above each graph with the dark gray shading representing the subjective night and the light gray shading representing subjective day.

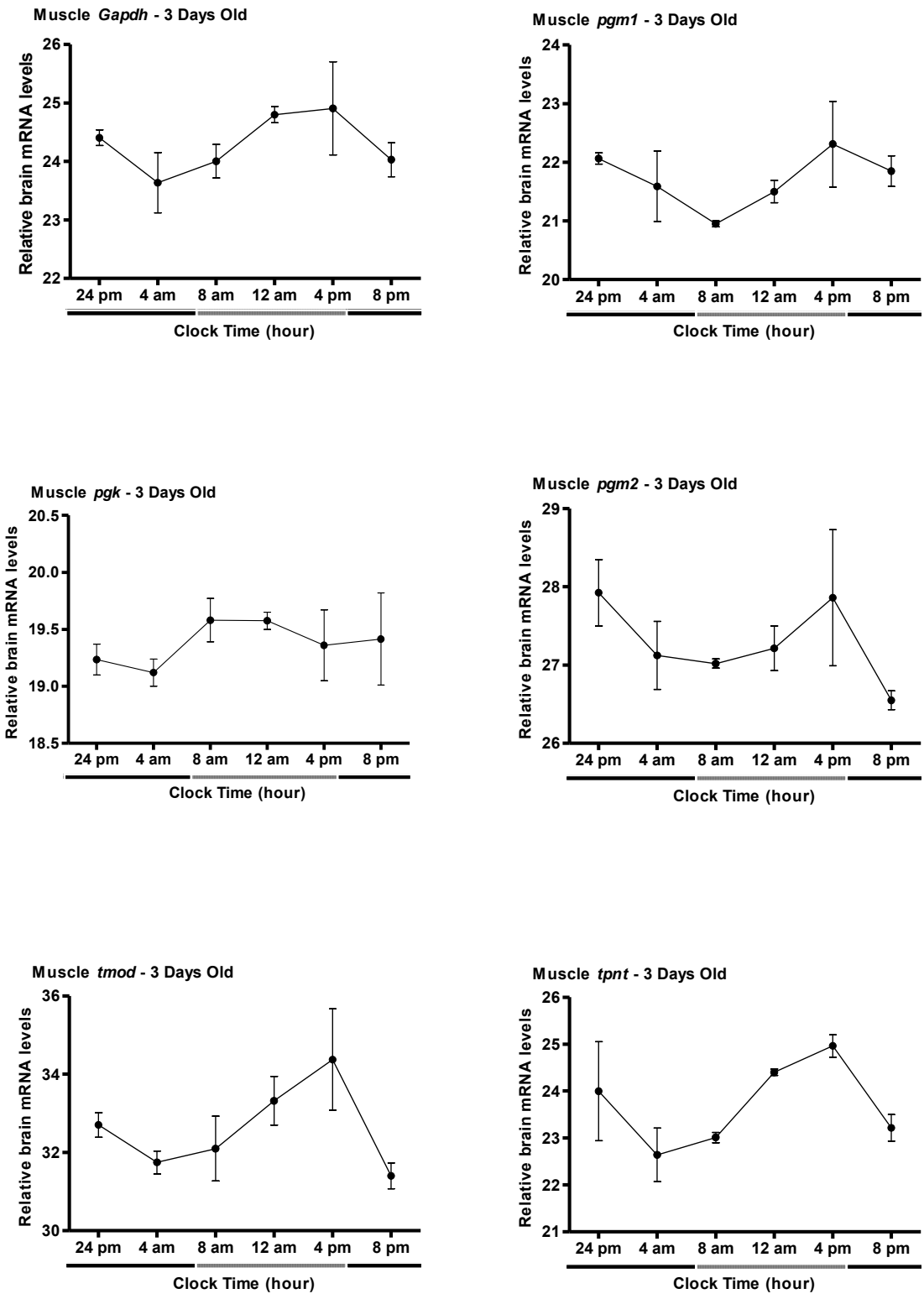


Fig.16. Twenty–four–hour profiles of 3-days-old honeybee flight muscle metabolic genes expression. Plotted are mRNA levels of candidate genes relative to the internal control gene *amRPS5*, in the honey bee flight muscle tissue, over a 24-h period in constant darkness. Each time point represents the mean ± SEM for the experiment (n=8). The environmental dark-light cycle before isolation into constant darkness (DD) is depicted above each graph with the dark gray shading representing the subjective night and the light gray shading representing subjective day.

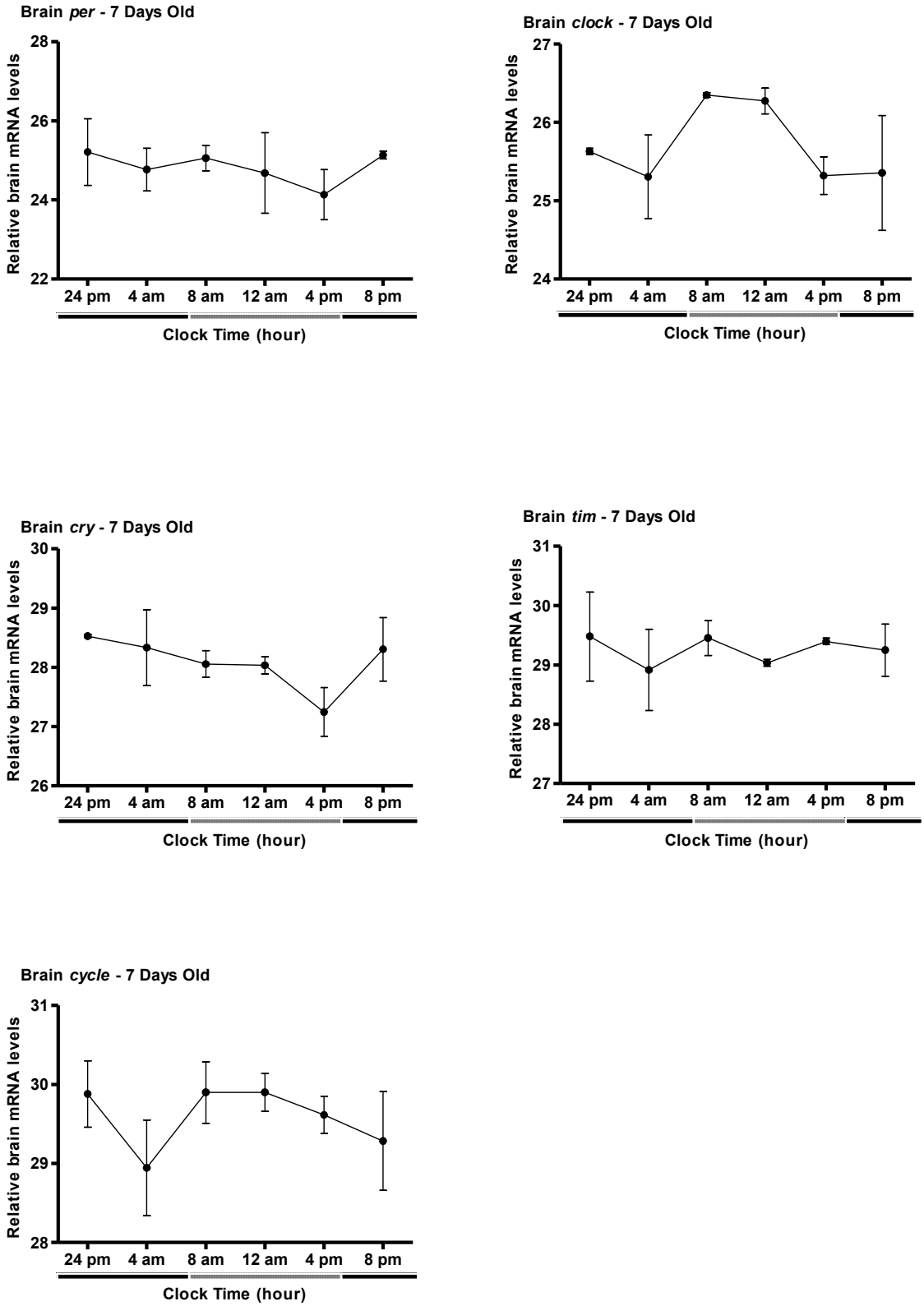


Fig.17. Twenty–four–hour profiles of 7-days-old honeybee brain core clock genes expression. Plotted are mRNA levels of candidate genes relative to the internal control gene *amRPS5*, in the honey bee brain tissue, over a 24-h period in constant darkness. Each time point represents the mean ± SEM for the experiment (n=8). The environmental dark-light cycle before isolation into constant darkness (DD) is depicted above each graph with the dark gray shading representing the subjective night and the light gray shading representing subjective day.

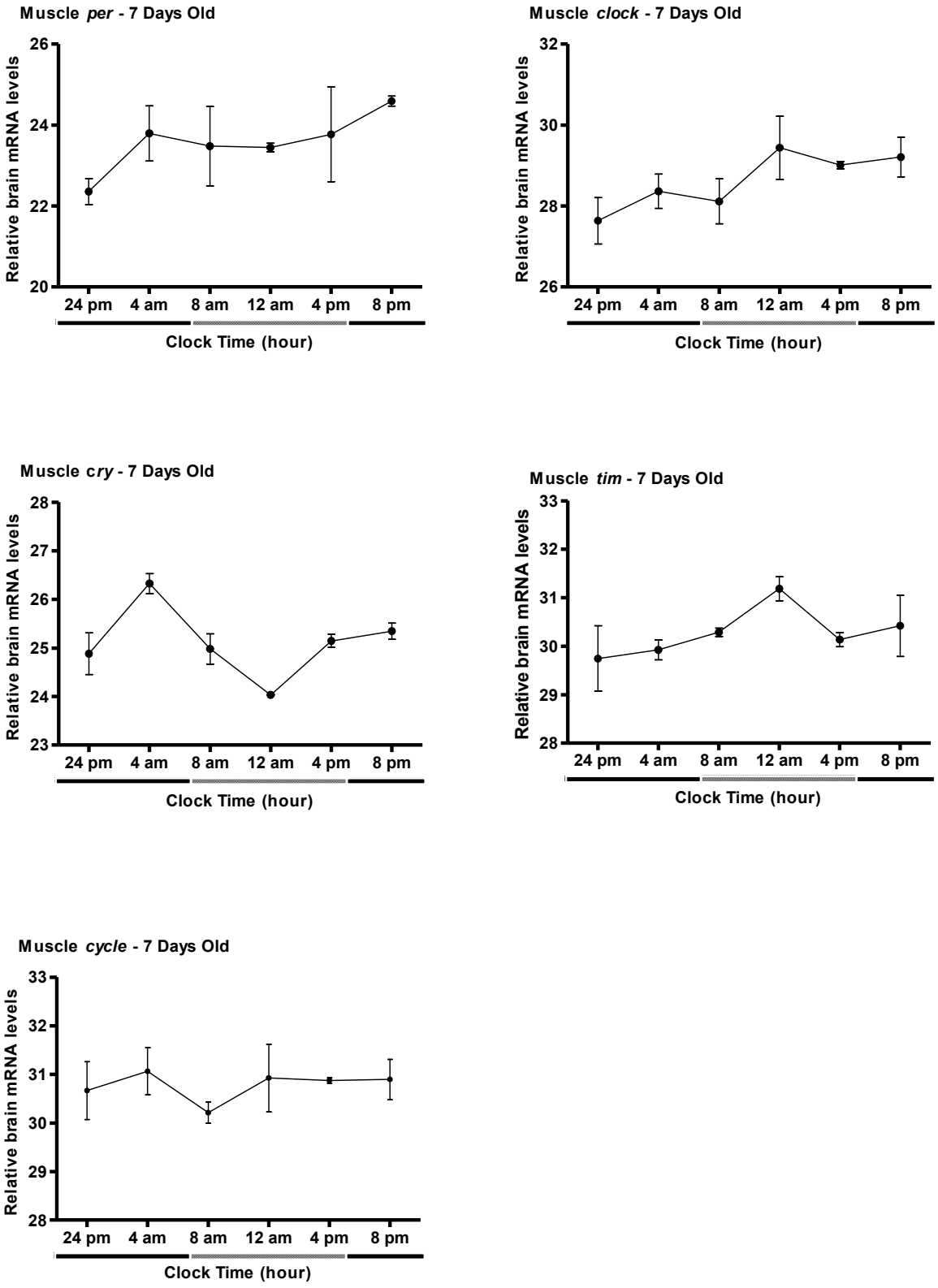


Fig.18. Twenty–four–hour profiles of 7-days-old honeybee flight muscle core clock genes expression. Plotted are mRNA levels of candidate genes relative to the internal control gene *amRPS5*, in the honey bee flight muscle tissue, over a 24-h period in constant darkness. Each time point represents the mean ± SEM for the experiment (n=8). The environmental dark-light cycle before isolation into constant darkness (DD) is depicted above each graph with the dark gray shading representing the subjective night and the light gray shading representing subjective day.

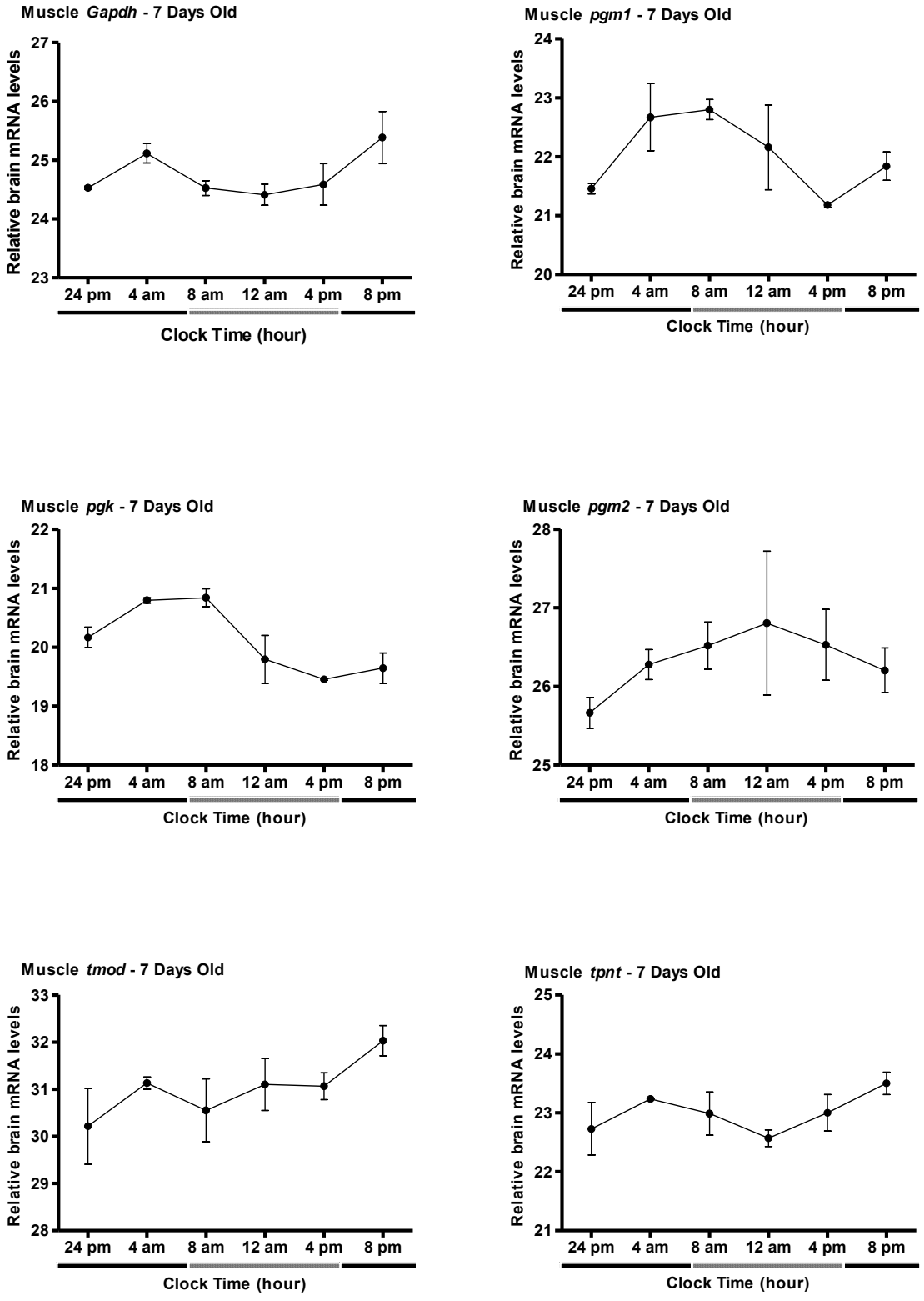


Fig.19. Twenty–four–hour profiles of 7-days-old honeybee flight muscle metabolic genes expression. Plotted are mRNA levels of candidate genes relative to the internal control gene *amRPS5*, in the honey bee flight muscle tissue, over a 24-h period in constant darkness. Each time point represents the mean ± SEM for the experiment (n=8). The environmental dark-light cycle before isolation into constant darkness (DD) is depicted above each graph with the dark gray shading representing the subjective night and the light gray shading representing subjective day.

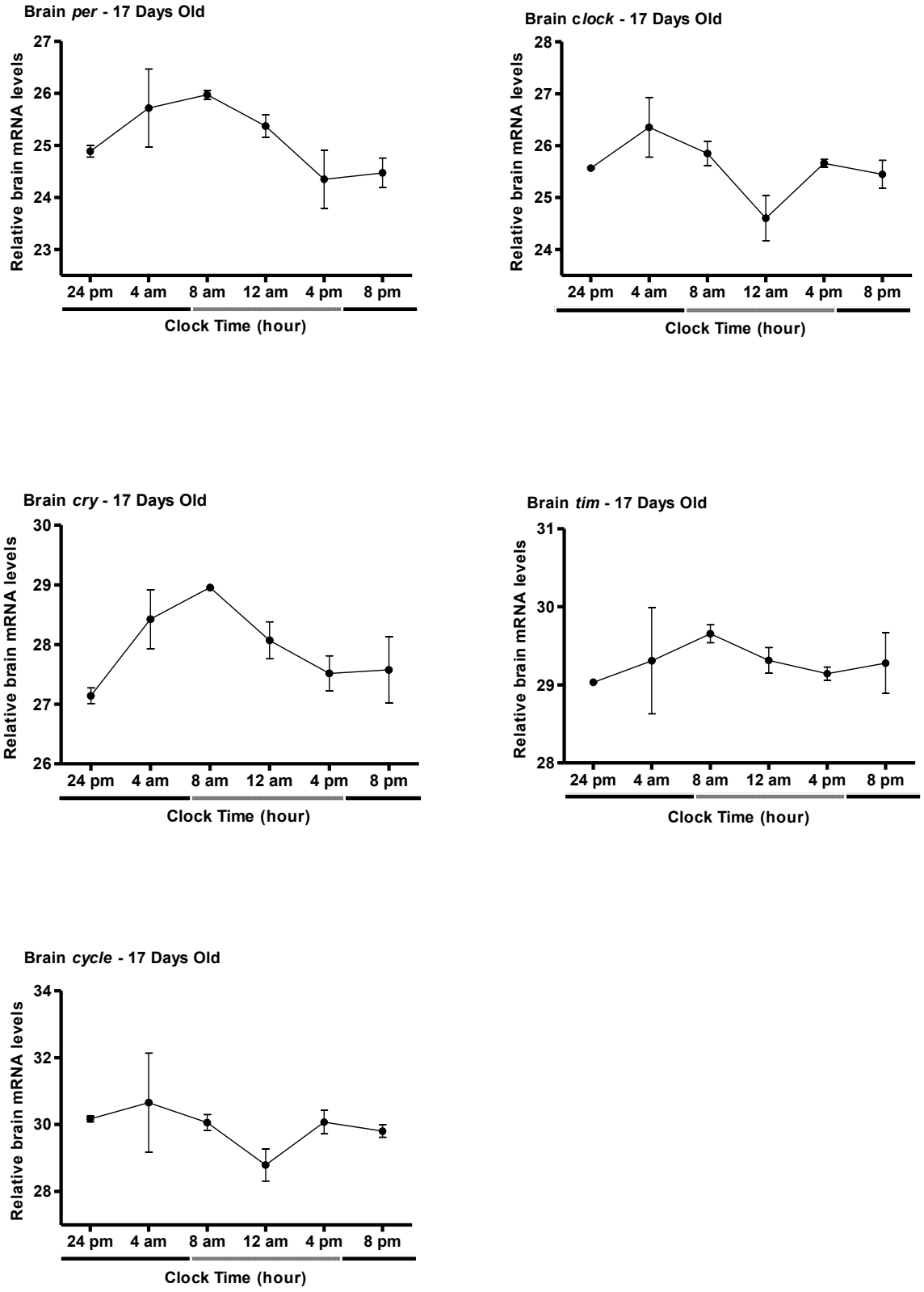


Fig.20. Twenty–four–hour profiles of 17-days-old honeybee brain core clock genes expression. Plotted are mRNA levels of candidate genes relative to the internal control gene *amRPS5*, in the honey bee brain tissue, over a 24-h period in constant darkness. Each time point represents the mean ± SEM for the experiment (n=8). The environmental dark-light cycle before isolation into constant darkness (DD) is depicted above each graph with the dark gray shading representing the subjective night and the light gray shading representing subjective day.

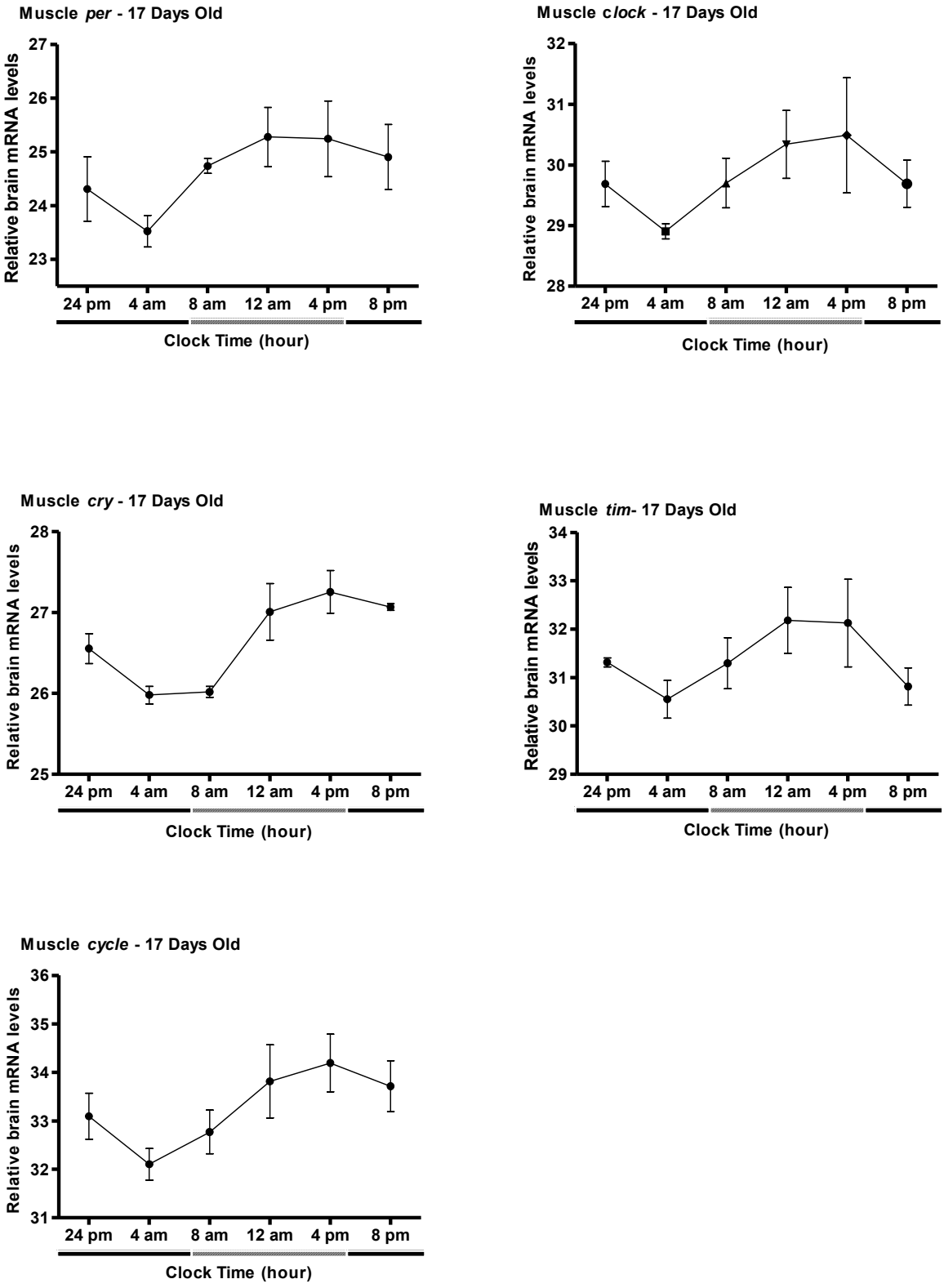


Fig.21. Twenty–four–hour profiles of 17-days-old honeybee flight muscle core clock genes expression. Plotted are mRNA levels of candidate genes relative to the internal control gene *amRPS5*, in the honey bee flight muscle tissue, over a 24-h period in constant darkness. Each time point represents the mean ± SEM for the experiment (n=8). The environmental dark-light cycle before isolation into constant darkness (DD) is depicted above each graph with the dark gray shading representing the subjective night and the light gray shading representing subjective day.

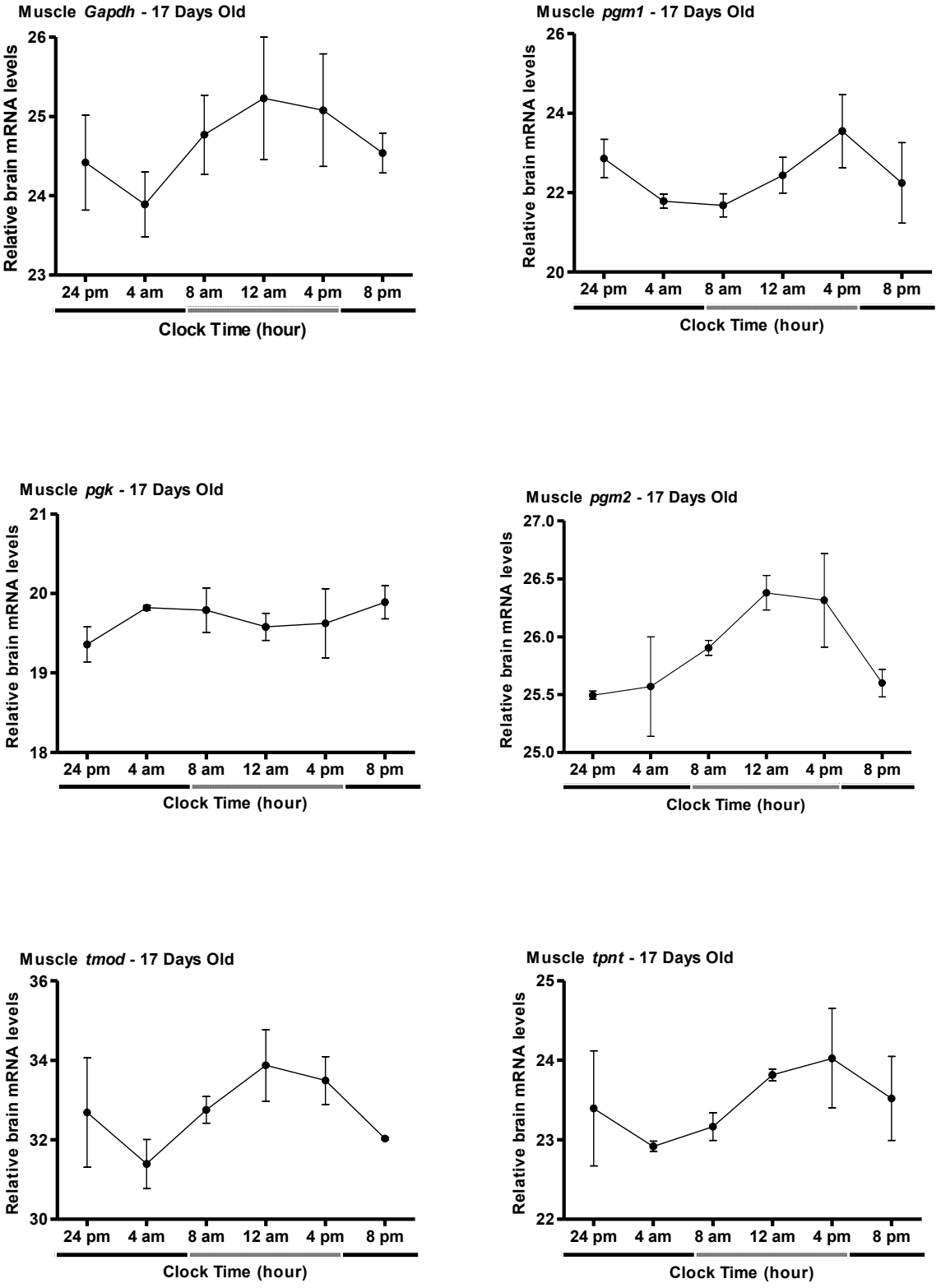
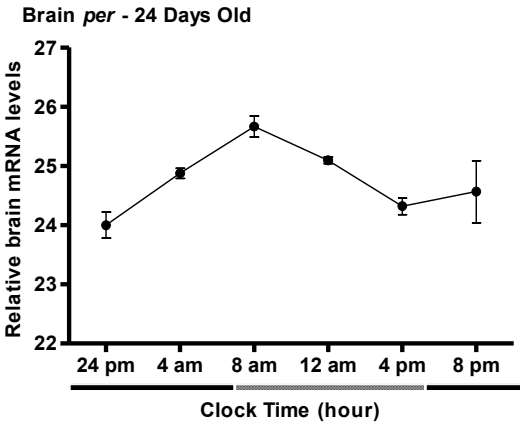
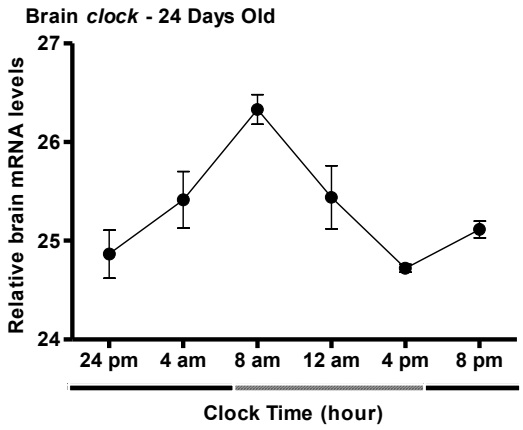
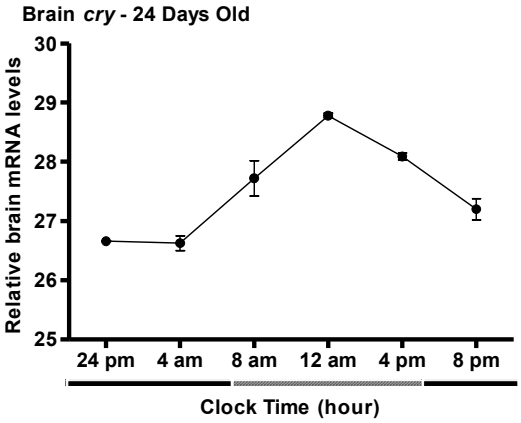
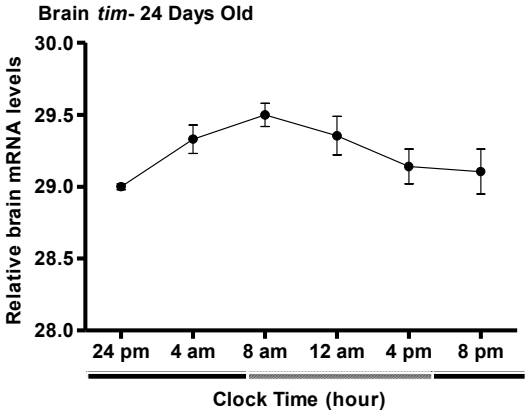


Fig.22. Twenty–four–hour profiles of 17-day-old honeybee flight muscle metabolic genes expression. Plotted are mRNA levels of candidate genes relative to the internal control gene *amRPS5*, in the honey bee flight muscle tissue, over a 24-h period in constant darkness. Each time point represents the mean ± SEM for the experiment (n=8). The environmental dark-light cycle before isolation into constant darkness (DD) is depicted above each graph with the dark gray shading representing the subjective night and the light gray shading representing subjective day.









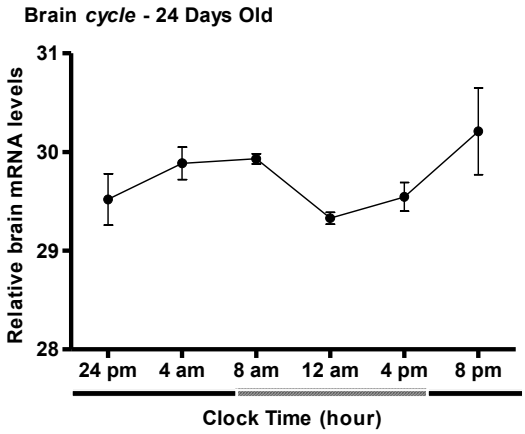
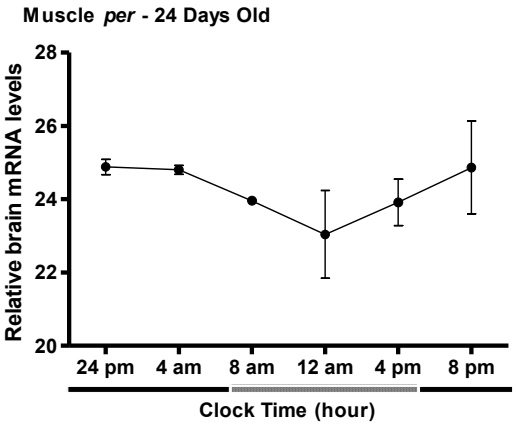
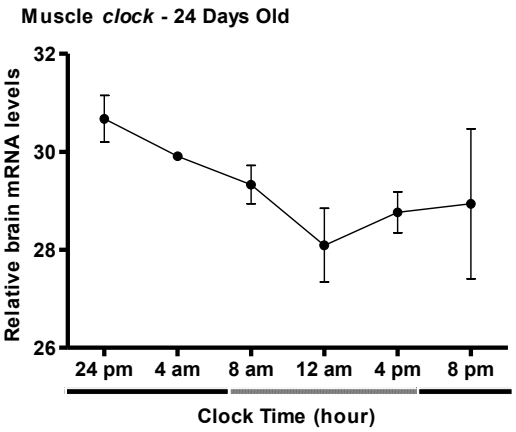
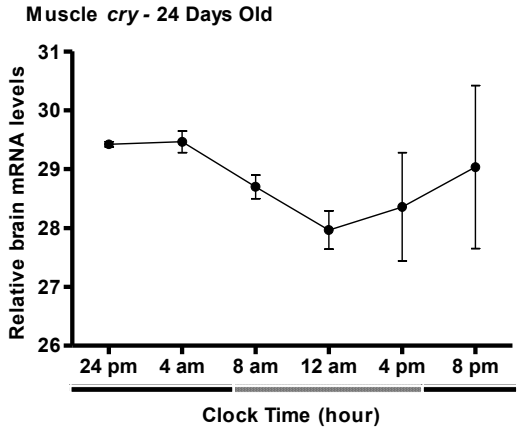
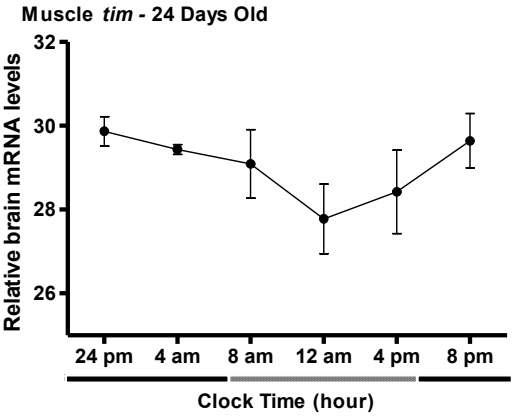


Fig.23. Twenty–four–hour profiles of 24-days-old honeybee brain core clock genes expression. Plotted are mRNA levels of candidate genes relative to the internal control gene *amRPS5*, in the honey bee brain tissue, over a 24-h period in constant darkness. Each time point represents the mean ± SEM for the experiment (n=8). The environmental dark-light cycle before isolation into constant darkness (DD) is depicted above each graph with the dark gray shading representing the subjective night and the light gray shading representing subjective day.









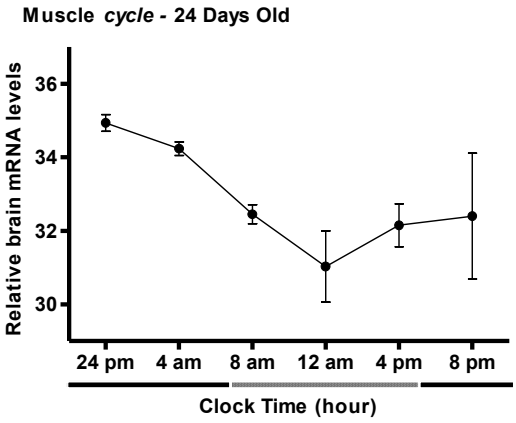


Fig.24. Twenty–four–hour profiles of 24-days-old honeybee flight muscle core clock genes expression. Plotted are mRNA levels of candidate genes relative to the internal control gene *amRPS5*, in the honey bee flight muscle tissue, over a 24-h period in constant darkness. Each time point represents the mean ± SEM for the experiment (n=8). The environmental dark-light cycle before isolation into constant darkness (DD) is depicted above each graph with the dark gray shading representing the subjective night and the light gray shading representing subjective day.

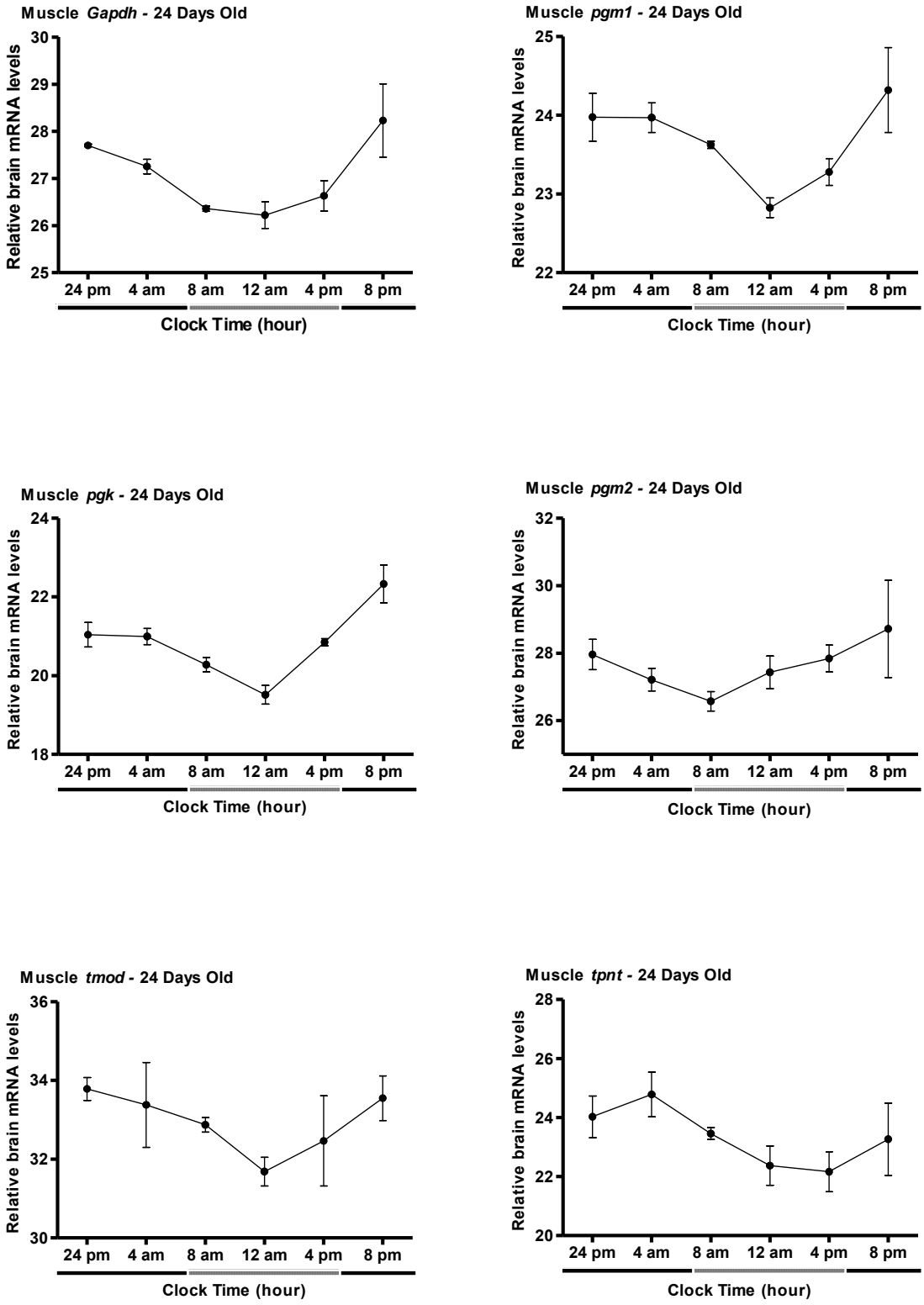


Fig.25. Twenty–four–hour profiles of 24-days-old honeybee flight muscle metabolic genes expression. Plotted are mRNA levels of candidate genes relative to the internal control gene *amRPS5*, in the honey bee flight muscle tissue, over a 24-h period in constant darkness. Each time point represents the mean ± SEM for the experiment (n=8). The environmental dark-light cycle before isolation into constant darkness (DD) is depicted above each graph with the dark gray shading representing the subjective night and the light gray shading representing subjective day.

At 1 day old of age, expression of core clock genes in brain and flight muscle has been found to be in perfect phase among tissues, with peak expression of four genes occurring at 0400 hours, at the beginning of the light phase, and minimum expression during the hour of lightness, at 1200 hours (Figs 1 and 2) over the 24 h period of time. Such synchronization in the pattern of genes expressions among tissues has been registered only for 2 clock genes, *per* and *clock,* at 3 days old (Figs 4 and 5); while the prominent 24 h oscillations in the gene expression previously registered in young individuals have been revealed to show a clear opposite amplitude between tissues for 17 and 24 ages old bees, as the transcript levels of the four clock genes was revealed to occur totally in antiphase in brain comparing to flight muscle tissue (Figs 10, 11, 13 and 14). Thus far, it seems that under constant conditions and in isolation, honeybees showed a sort of synchronization in the rhythm features in core clock genes expressed in brain and flight muscle, since they emerged. It is only later, when they relatively aged, that the situation tended to be inversed, showing more discrepancy in the trend of the oscillations in gene expression among central and peripheral tissues level.

3.3.2. Daily variation in the expression of metabolic genes in flight muscle tissue according to age development of honeybee

The rhythmic expression of the genes involved in flight muscle metabolism of honeybees showed a clear circadian pattern of oscillation over the 24 h period of time, although transcript levels was weakly significant in major number of genes (see tab. 3). Indeed, the variation in expression levels over time for the metabolic genes, was a result of balancing between the positive and negative regulators of the core clock feedback loop that oscillate in muscle tissue (Figs 3, 6, 9, 12 and 15). It is noteworthy that the expression levels of all the metabolic genes were found to be in striking phase with those of the main core clock genes expressed at the level of the muscle tissue, independently of the age of the isolated honeybees, and consequently less in phase with the level expression of the latest in the brain, obviously during relative oldest ages, as detailed above.

3.4 Discussion

Our interest to study in honeybee the clock and metabolic gene expression in the flight muscle tissue, compared to the brain, derived from the hypothesis that, in these insects, the circadian clock might be endogenously regulated, in an independent manner, and may play an important role, during the ontogenetic development, in setting the phase to some metabolic events which are essential to flight muscle capacity.

Results from the current study has shown for the first time that the core molecular clock components in the honeybee flight-muscle are rhythmically expressed, under free-running conditions, and that the 24h temporal expression of such machinery system is already active in the newly born workers. Thus, and independently from bee's age, *per* and *cry* have shown to peak in phase, in relation with their primary roles in generating endogenous rhythms. For these two genes transcripts, a similar temporal expression profile has been reported previously in honeybee (Rubin *et al.*, 2006). The expression of the core clock genes *tim, cyc* and *clock* mRNA, although had registered a weak significance (anyway with yielded *P* values tending to approach significance; see tab. 3) through different age matched honeybees, their oscillating transcription waveform have shown a striking circadian trend in the analyzed tissue, which could suggest that both the PER-TIM and CLK-CYC feedback loops may exist in the flight muscle of early youngest and later oldest honeybees.

Recently, in studies realized especially on mammals, it was reported that the skeletal muscle tissue contains a peripheral clock, as verified in species like mice, horse or zebrafish (McCarthy *et al.*, 2007; Martin *et al.*, 2010; Amaral & Johnston, 2012). By contrast, to date, the only studies examining temporal expression profile of the core clock genes overall honeybee body have been focusing only on the brain (reviewed in Bloch, 2010).

Though still in presence of a relatively limited set of results, which strongly need further analysis and deeper insights, an attempt will be made in the following pages to elaborate some summary considerations which may derive from the experiences realized up to now.

At this point, and with reference to the molecular and phylogenetic analysis that revealed mammalian like clockwork in the honeybee (Rubin *et al.*, 2006), we have determined prevalently that, in the honeybee, the circadian cycling of core clock genes RNA, although tending to persist

under free running conditions, showed to follow unusual temporal patterns of expression from those ones yet pre-established in the brain. For example, and contrary to what was reported by Rubin *et al.* (2006), our studies have shown that *amclock* RNA levels did vary over time and *amcyc* oscillated with high amplitude in muscles of 1 and 3 days old bees and almost in phase with *amper* and *amcry*. For some aspects, these patterns which have been obtained seem to be distinct either for *Drosophila* or the honeybee. At this level, and according to multiple explanations that could converge with our data, we are favorable to relate such peculiar aspects to presumable influences deriving from our protocol design, in which we proceed to sample honeybees that were caged individually since emergence and under DD illumination (to avoid light–regulated genes), rather than to collect individuals in constant darkness, after having entrained the experimental colonies for a number of days in 12h light / 12h dark (LD) illumination regime (as done by Rubin *et al.*, 2006). Recent studies, realized on wild type (WT) of *Drosophila* under constant conditions of darkness (DD) and completed by using microarray, have revealed strong oscillations of various genes which are well known as implicated in circadian rhythms, including *period* (*per)*, *timeless (tim), clock (clk), vrille (vri), cryptochrome (cry*) and *takeout* (*to*), that were found to oscillate according to a high amplitude and same phase (Ueda *et al.*, 2002). Another line of evidence could be taken into consideration, which essentially relies to the possible weak circadian fluctuation of any of the molecular components that occurred in honeybee body tissues, under impact of dumping, as it is known that many circadian features weaken as a function of time in constant darkness (McDonald & Rosbash, 2001). This result encourages further investigations of body-wide orchestration of circadian rhythms that could provide a molecular explanation for the tissue-related profiles of periodic gene expression obtained in youngest versus oldest individuals in honeybee. Interestingly, such findings have revealed an evident age-related change from a striking similarities to sharp differences in the temporal expression pattern of core clock genes between brain and peripheral flight-muscle tissue. Thus, we propose a subdivision of the five experimented groups of different aged bees into 2 clusters, according to the overall organization of the cycling genes RNA expression patterns. The first cluster corresponds to the bees that belong to the youngest ages (1 and 3 days old), in which the flight muscle and brain oscillators behave similarly, while the second cluster includes the remaining groups of older aged bees (especially 17 and 24 days old), that exhibited the opposite behavior between cycling oscillators. Such classification, though yet not enough to justify the autonomy of the functioning flight-muscle oscillator, might suggest somewhat differences between the mechanisms for coupling the autonomous oscillators

(Cluster 2) from those for non autonomous oscillators (Cluster 1) in the case of social insects. In any case, and for the first time at the peripheral level, such data provide further support for the natural plasticity in circadian rhythms, that it is somehow associated with reorganization of the internal clockwork in honeybees, following the transition from nursing to foraging behavior.

The comparisons of the molecular analysis between the two above mentioned prevalent clusters, make us tempting to investigate the entrainment signals of relevance in modulating profoundly the exhibited molecular plasticity in the overall clockwork of bees, over their life span. The hive environment is considered to be the primary factor to influence the ontogeny of the circadian rhythms of individuals; and it is thought to be of major importance in tuning the social synchronization of worker bees. Under constant conditions, the 1 day old bees, just after emergence, exhibited a robust circadian rhythm in overall gene expressions in both tissues. This result is intriguing, as it contrasts with previous studies that supported the evidence that bees typically develop overt rhythm, with a FRP of about 24h, not earlier than their first days after eclosion (Spangler, 1972; Bloch *et al.*, 2002; Toma *et al.*, 2000; Meshi & Bloch, 2007). Thus, in addition to provide evidences of an endogenous circadian clockwork that function before rhythms are overtly expressed, such data could be in part supportive of our hypothesis that multiple oscillators exist within and outside the brain of honeybees, whose phases are set by exogenously driven components, with special and social ones. Inside the hive, synchronization of the pupae is probably regulated by QMP communication process; and the queen entrainment could allow future workers to maintain and to be in phase with the timing of the outside-the-hive sisters until they are ready to begin independent lives, as they are never directly exposed to environmental day-night cycles. In rodents, maternal cues have been demonstrated to exert powerful effect on the neonatal clock, as a 24h maternal cycles has been exhibited either during absence of the original mother or in presence of a foster one (as reviewed by Weinert., 2005). This hypothesis, although justify recent results that demonstrated the rapid, almost instant, overt rhythm activity, with a phase entrained to the hive environment, regardless of the time when the nurse-bee is removed from the hive (Shemesh *et al.*, 2010), could less explain the opposite wave form and phase trends of the molecular oscillations between the same aged-nurses sampled either under natural (in-hive sampling) or under constant conditions (laboratory sampling), at least at peripheral level. Additional studies are needed to further test the molecular mechanism that underlies the entrainment. However, and given importance to worker-bees related data, it should be noted that the transition from nursing to foraging might be associated with plasticity in the

regulation of the circadian rhythm overall behavior and molecular oscillations, which is itself influenced by the social context. Under constant darkness, the isolated 3 days old bees have exhibited a robust cycling that was in synchrony in both tissues, in concert with the free flying forager's temporal patterns of gene expression (as derived from the field research). In contrast, and once placed individually in cages under the same constant laboratory conditions, pre-forager and forager old sisters (17 and 24 days old), unlikely and only at brain level, exhibited a shifting to "in-hive" nurse's oscillating pattern of gene expression; while, on the contrary, from flight-muscle tissue, a predominant daily pattern in gene clock oscillations has been more revealed. These adjustments are analogous to what has been observed from precocious foragers (young workers) or reverted nurses (typical aged bees) that have been obtained as a consequence of an unbalanced colony age demography manipulated colonies (Bloch & Robinson, 2001). Such strategically indirect social entrainment (absence of conspecifics) of the individual endogenous rhythms would have some selective advantages for the species.

Assuming that the variation in overt molecular rhythms is indeed circadian (internal), the next question is to define which internal signals are responsible in honeybee for the phase entrainment of the peripheral core oscillator and for its phase shifting during the transition from nursing to foraging. Contributing to this outcome, and based on new evidences that started to link the effect of the internal nutritional physiology on the global gene expression in the brain and the regulation of the foraging ontogeny (Toth *et al.*, 2005; Rodriguez-Zas *et al.*, 2012), we originally proposed to link our data regarding the molecular clockwork analysis of rhythms registered in the flight muscle somewhere to the mechanisms underlying individual energy metabolism. Contrary to many others peripheral tissues, the investigation of the specific entrainment factors for skeletal muscle is still highly speculative, due to the important role it plays in either whole body metabolism or in behavioral activities. Despite this evidence, recent results shed light on the ability undergone by the feeding regulation, as an entrainment signal, on tissue-specific clock gene expression in many mammals peripheral tissues, without any involvement of the central pacemaker in the brain (Xu *et al.*, 2008). For example, without any established results, noticeable physiological changes like altered circadian profiles of plasma glucose, insulin, corticosterone or free fatty acids have been associated with restricted feeding regimens (Dyar, 2009) and suggested to be of impact in altering circadian gene expression at the level of skeletal muscle physiology. Our studies presented a lack of information that could increase evidences about the feeding impact on the global gene expression rhythms in peripheral muscle clock; however, substantial literature has uncovered the

modulation of core oscillator genes expression through food availability and especially the restricted feeding schedule (Damiola *et al.*, 2000; Stokkan *et al.*, 2001). In fact, investigating nocturnal rodents response to food availability during light phase, revealed a phase shifting of core oscillator gene expression in different peripheral clocks (Damiola *et al.*, 2000; Stokkan *et al.*, 2001), in peculiar skeletal muscle (Dyar, 2009), without affecting the central oscillator. This results supported the suggested role played by the metabolic factors, rather than activity, in entraining rhythmic clock gene expression in skeletal muscle. It is well-known that honeybee is highly implicated in an evolutionary regulatory process interfering on the individual nutritional status (Toth *et al.*, 2005; Ament *et al.*, 2011), in phase with their lifestyle transition from in-hive tasks to foraging requirements. Thus, it is possible that the nutritional status of bees, before and during their isolation under constant darkness, has had some mediated effect on the regulated plasticity in the circadian expression of clock and putative clock-controlled genes, especially in flight muscle of bees. Moreover, although each individuals has been fed *ad libitum*, either the quality or the way of food taken might have impacted muscle clock behavior, even for short term analyzed data. This interpretation has to include other separate pathways that involve social mediation (food exchange) in causing part of the resulting temporal expression pathways of flight muscle molecular responses. Such suggestions are consistent with recent findings that enumerated various effects of nutrition and social inhibition on behavioral maturation in honey bees (Toth *et al.,* 2005). At our level, and especially towards this direction, further investigations are worth being developed in the next future.

4. Conclusions

This study breaks new ground as it is the first of its kind to demonstrate the existence of an endogenous peripheral tissue clock in a social insect: the honeybee. Therefore, the study aimed at investigating the intricate network of molecular clocks in the body periphery with emphasis on the flight muscle tissue, to extend the knowledge of whether circadian clockwork might exhibit profound context –dependent plasticity, beyond the well-known brain – located clock containing tissue. The implications are that bee worker's flight muscle undergoes rhythmical 24 h regulation in gene expression, which temporal cycling pattern, in turn differed significantly in LD and under DD, according to functional mechanisms deriving from the social entrainment. In addition, because specific individual behavioral response to social context requirement, is a coordinated response of multiple physiological processes, the identification of several clock controlled genes and clock regulated metabolic processes of muscle functions, to our knowledge, are the strongest evidence to date supporting the idea that specific flight muscle peripheral clock may exist in the circadian system of such complex insect and functions in a tissue – specific manner, in correlation with their sensitivity to external entraining signals. Although intuitively not obvious, it is conceivable that a collection of autonomous entrained clocks could fine-tune the specific behavioral and physiological state of a bee, that accompanied the broad range of social lifestyle it exhibits along its life span. For example, at the time when locomotor activity lacks entrainment from the clock in young nurse's brain, an interesting clock via its neuronal and hormonal outputs, may stimulates oscillations in the expression of clock genes in other cells and tissues, involved in physiological processes. This seems to be no simple hypothesis to confirm, especially in the case of social insects, which are subject to complex natural society, where the rhythms of each individual both influence and are sensitive to the rhythms of others in the "super-organism" colony. Nevertheless, exciting future opportunities to discover others CCGs and clock regulated processes in the honeybee model, exposed to a far more functional heterogeneous environment outside and inside the hive through pertinent experimental protocols, might add meaningful progresses in probing the genuine circadian organization in our model system.

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