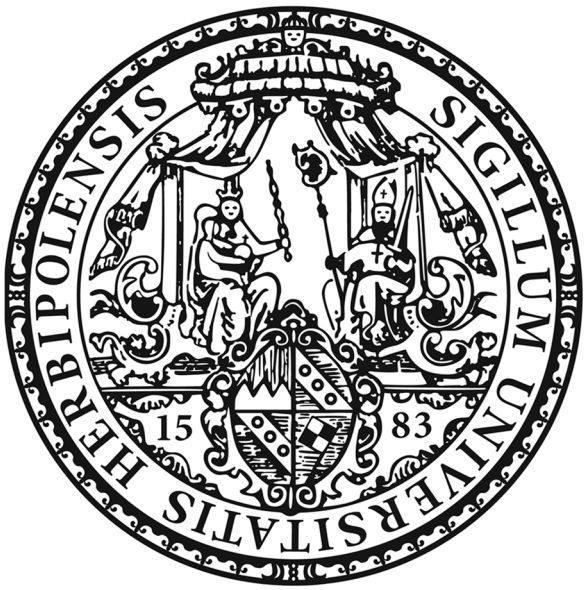
A Comparison of the circadian clock of highly social bees (*Apis mellifera*) and solitary bees (*Osmia spec.*):

Circadian clock development, behavioral rhythms and neuroanatomical characterization of two central clock components (PER and PDF)



Ein Vergleich der Inneren Uhr von sozialen Bienen (*Apis mellifera*) und solitären Bienen (*Osmia spec.*):

Entwicklung der circadianen Uhr, Verhaltensrhythmen und neuroanatomische Beschreibung von zwei zentralen Uhr Komponenten (PER und PDF)

Doctoral thesis for a doctoral degree at the Graduate School of Life Sciences,

Julius-Maximilians-Universität Würzburg,

Section: Integrative Biology

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Würzburg, November 2017

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Für meinen Papa

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Zusammenfassung

Bienen, sowie viele andere Organismen, evolvierten eine innere circadiane Uhr, die es ihnen ermöglicht, tägliche Umweltveränderungen voraus zu sehen und ihre Foragierflüge zu Tageszeiten durchzuführen, wenn sie möglichst viele Blüten besuchen können. Es zeigte sich, dass der soziale Lebensstil der Honigbiene Einfluss auf das rhythmische Verhalten der Ammenbienen hat, die während der Brutpflege keinen täglichen Rhythmus im Verhalten aufweisen. Sammlerbienen auf der anderen Seite zeigen ein stark rhythmisches Verhalten. Solitäre Bienen, wie die Mauerbiene, betreiben keine Brutpflege und leben nicht in einer Staatengemeinschaft, aber sind den gleichen Umweltveränderungen ausgesetzt. Nicht nur Lebensstil, sondern auch Entwicklung und Lebenszyklus unterscheiden sich zwischen Honig- und Mauerbienen. Mauerbienen überwintern als adulte Insekten in einem Kokon bis sie im Frühjahr schlüpfen. Honigbienen durchleben keine Diapause und schlüpfen nach wenigen Wochen der Entwicklung im Bienenstock. In meiner Dissertation vergleiche ich die circadiane Uhr von sozialen Honigbienen (*Apis mellifera*) und solitären Mauerbienen (*Osmia bicornis* und *Osmia cornuta*) auf Ebene der Neuroanatomie und das durch die innere Uhr verursachte rhythmische Verhalten.

Erstens charakterisierte ich detailliert die Lage der circadianen Uhr im Gehirn von Honig- und Mauerbiene anhand des Expressionsmusters von zwei Uhrkomponenten. Diese sind das Uhrprotein PERIOD (PER) und das Neuropeptid Pigment Dispersing Factor (PDF). PER wird exprimiert in lateralen Neuronen-Gruppen (die wir laterale Neurone 1 und 2 nannten: LN_1 und LN_2) und dorsalen Neuronen-Gruppen (benannt dorsal laterale Neurone und dorsale Neurone: DLN und DN), sowie in vielen Gliazellen und Fotorezeptorzellen. Dieses Expressionsmuster liegt ähnlich in anderen Insektengruppen vor und deutet auf einen Grundbauplan der Inneren Uhr im Gehirn von Insekten hin. In der LN_2 Neuronen-Gruppe, deren Zellkörper im lateralen Gehirn liegen, sind PER und PDF in den gleichen Zellen co-lokalisiert. Diese Zellen bilden ein komplexes Netzwerk aus Verzweigungen durch das gesamte Gehirn und liefern damit die perfekte Infrastruktur, um Zeitinformation an Gehirnregionen weiterzuleiten, die komplexe Verhaltensweisen, wie Sonnenkompass-Orientierung und Zeitgedächtnis, steuern. Alle PDF Neuriten laufen in einer anterior zur Lobula liegenden Region zusammen (sie wurde ALO, anterio-lobular PDF Knotenpunkt, genannt). Dieser Knotenpunkt ist in anderen Insekten mit der Medulla assoziiert und wird akzessorische Medulla (AME) genannt. Wenige PDF Zellen bilden bereits im frühen Larvalstadium diesen ALO und die Zellzahl sowie die Komplexität des Netzwerks wächst die gesamte Entwicklung der Honigbiene hindurch. Dabei werden zuerst die dorsalen Gehirnregionen von PDF Neuronen innerviert und in der späteren Larvalentwicklung wachsen die Neurite lateral in Richtung der optischen Loben und des Zentralgehirns. Das generelle Expressionsmuster von PER und PDF in adulten sozialen und solitären Bienen ähnelt sich stark, aber ich identifizierte kleine Unterschiede in der PDF Netzwerkdichte im posterioren Protocerebrum und in der Lamina. Diese könnten mit der Evolution von sozialen Bienen assoziiert sein.

Zweitens entwickelte und etablierte ich eine Methode, Lokomotionsrhythmen von individuellen Bienen im Labor aufzunehmen, die in Kontakt mit einem Miniaturvolk standen. Diese Methode enthüllte neue Aspekte der sozialen Synchronisation unter Honigbienen und des Überlebens von jungen Bienen, die

indirekten sozialen Kontakt zu dem Miniaturvolk hatten (Trophalaxis war nicht möglich). Für Mauerbienen etablierte ich eine Methode Schlupf- und lokomotorische Aktivitätsrhythmik aufzuzeichnen und konnte damit zeigen, dass tägliche Rhythmen im Schlupf durch Synchronisation der circadianen Uhr in Mauerbienen durch Tagestemperatur-Zyklen erzielt werden kann. Des Weiteren präsentiere ich die ersten lokomotorischen Aktivitätsrhythmen von solitären Bienen, die sofort nach ihrem Schlupf einen starken circadianen Rhythmus im Verhalten aufwiesen. Honigbienen brauchten in meinen Experimenten mehrere Tage, um circadiane Rhythmen in Lokomotion zu entwickeln. Ich erstellte die Hypothese, dass Honigbienen zum Zeitpunkt des Schlupfes im Bienenvolk ein noch nicht vollständig ausgereiftes circadianes System besitzen, während solitäre Bienen, die ohne den Schutz eines Volkes sind, direkt nach dem Schlupf eine vollständig ausgereifte Uhr brauchen. Mehrere Hinweise in Publikationen und Vorversuchen unterstützen meine Hypothese. Zukünftige Studien der Entwicklung des PDF Neuronen-Netzwerkes in solitären Bienen unterschiedlicher Entwicklungsstufen könnten dies nachweisen.

Summary

Bees, like many other organisms, evolved an endogenous circadian clock, which enables them to foresee daily environmental changes and exactly time foraging flights to periods of floral resource availability. The social lifestyle of a honey bee colony has been shown to influence circadian behavior in nurse bees, which do not exhibit rhythmic behavior when they are nursing. On the other hand, forager bees display strong circadian rhythms. Solitary bees, like the mason bee, do not nurse their offspring and do not live in hive communities, but face the same daily environmental changes as honey bees. Besides their lifestyle mason and honey bees differ in their development and life history, because mason bees overwinter after eclosion as adults in their cocoons until they emerge in spring. Honey bees do not undergo diapause and have a relatively short development of a few weeks until they emerge. In my thesis, I present a comparison of the circadian clock of social honey bees (*Apis mellifera*) and solitary mason bees (*Osmia bicornis* and *Osmia cornuta*) on the neuroanatomical level and behavioral output level.

I firstly characterized in detail the localization of the circadian clock in the bee brain via the expression pattern of two clock components, namely the clock protein PERIOD (PER) and the neuropeptide Pigment Dispersing Factor (PDF), in the brain of honey bee and mason bee. PER is localized in lateral neuron clusters (which we called lateral neurons 1 and 2: LN_1 and LN_2) and dorsal neuron clusters (we called dorsal lateral neurons and dorsal neurons: DLN, DN), many glia cells and photoreceptor cells. This expression pattern is similar to the one in other insect species and indicates a common ground plan of clock cells among insects. In the LN_2 neuron cluster with cell bodies located in the lateral brain, PER is co-expressed with PDF. These cells build a complex arborization network throughout the brain and provide the perfect structure to convey time information to brain centers, where complex behavior, e.g. sun-compass orientation and time memory, is controlled. The PDF arborizations centralize in a dense network (we named it anterio-lobular PDF hub: ALO) which is located in front of the lobula. In other insects, this fiber center is associated with the medulla (accessory medulla: AME). Few PDF

cells build the ALO already in very early larval development and the cell number and complexity of the network grows throughout honey bee development. Thereby, dorsal regions are innervated first by PDF fibers and, in late larval development, the fibers grow laterally to the optic lobe and central brain. The overall expression pattern of PER and PDF are similar in adult social and solitary bees, but I found a few differences in the PDF network density in the posterior protocerebrum and the lamina, which may be associated with evolution of sociality in bees.

Secondly, I monitored activity rhythms, for which I developed and established a device to monitor locomotor activity rhythms of individual honey bees with contact to a mini colony in the laboratory. This revealed new aspects of social synchronization and survival of young bees with indirect social contact to the mini colony (no trophalaxis was possible). For mason bees, I established a method to monitor emergence and locomotor activity rhythms and I could show that circadian emergence rhythms are entrainable by daily temperature cycles. Furthermore, I present the first locomotor activity rhythms of solitary bees, which show strong circadian rhythms in their behavior right after emergence. Honey bees needed several days to develop circadian locomotor rhythms in my experiments. I hypothesized that honey bees do not emerge with a fully matured circadian system in the hive, while solitary bees, without the protection of a colony, would need a fully matured circadian clock right away after emergence. Several indices in published work and preliminary studies support my hypothesis and future studies on PDF expression in different developmental stages in solitary bees may provide hard evidence.

1. Introduction

In the following section, I will shortly introduce basic characteristics of endogenous clocks in general and address circadian time keeping mechanisms in different insects. Afterwards I will elucidate the current knowledge on the circadian clock and rhythms in bees with focus on the bee species I work with in my thesis: social honey bee (*Apis mellifera*) and solitary mason bee (*Osmia bicornis* and *Osmia cornuta*). Furthermore, I will present the different lifestyles and life history of these two bee types and introduce the two clock components PER (PERIOD) and PDF (Pigment Dispersing Factor) I investigated in the thesis. Their expression pattern marks the localization of the circadian clock in the bee brain like in other insects demonstrated before.

1.1. A rhythmic world

We experience different rhythmical changes in our world. Day and night alternate because of the rotation of the earth around its own axis, the seasons pass by in the course of one year, our oceans rise and fall and the moon's shape changes in a month. To cope with these changes organisms evolved an endogenous clock, which enables them to predict rhythmic events and react at the right time. These clocks tick with a speed roughly matching the rhythms preset by the environment. Therefore, they are called circadian (~24 h), circannual (~ year), circatidal (~ 12.4 h) or circalunar (~29.5 d). Although there are many different rhythms in our world the circadian clock is one of the best studied endogenous clocks so far and will be of central interest in this thesis.

1.2. The circadian clock

The first reports of the existence of daily rhythms where made in the 18th century. In 1729 the astronomer Jean Jaques De Mairan observed circadian rhythms in leave movements of mimosa (*Mimosa pudica*) without the influence of light (de Mairan, 1729). Different plant species have specialized flowering to certain times of the day as Carl Linneaus shows in his "flower clock" (Linnaeus, 1751). The internalization of daily reoccurring variations seems highly adaptive (Vaze and Sharma, 2013) and indeed the circadian clock evolved in organisms as different as bacteria, plants and animals (Aschoff and Pohl, 1978; Bell-Pedersen et al., 2005). The three basic components of circadian time keeping i.e. input, oscillator(s) and output, are consistent throughout organisms, but exact mechanisms differ (Edery, 2000) (**Fig. 1**).

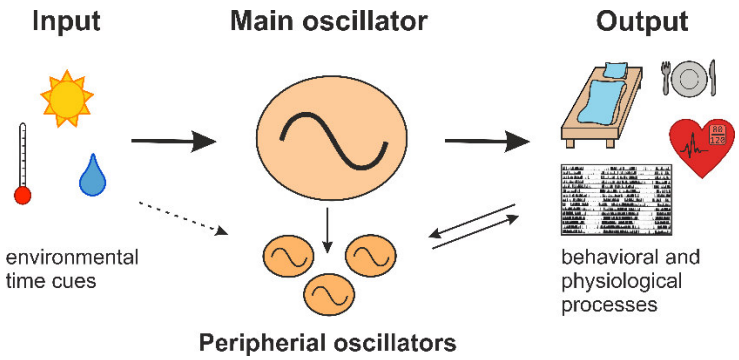

Figure 1: Basic elements of circadian rhythm generation.
The input signals from the environment entrain the molecular oscillator of the clock. The main oscillator in the brain (and indirectly the peripheral body oscillators) drive daily rhythms in behavioral and physiological processes like sleep, locomotor activity or blood pressure.

The circadian clock (main oscillator in the brain and peripheral oscillators in the body) is based on transcriptional/translational feedback loops (TTFL), in which clock genes inhibit their own transcription, which causes circadian oscillations in protein levels. These oscillations drive daily rhythms in behavior and physiological processes (Allada and Chung, 2010; Mohawk et al., 2012). The clock is synchronized (entrained) to the environment by input signals such as oscillating light or temperature. Without environmental time cues ("Zeitgebers") the clock runs free with its own period (τ or FRP), whereby τ differs with species, genotype and even individual state (Aschoff, 1981; Pittendrigh, 1960; Roenneberg et al., 2003). The clock is in different organisms more or less temperature compensated (Aschoff, 1981; Pittendrigh, 1960).

The main oscillator (core clock) in animals consists of a neuronal network in the brain, but there are also peripheral oscillators (body clocks) in other organs, which are synchronized by the main oscillator either directly via nervous or humoral regulation or indirectly by output rhythms (Dibner et al., 2010) (**Fig.1**). When uncoupled from the core clock peripheral oscillators can also be directly synchronized by environmental times cues like for example food availability, temperature or light cycles (Brown et al., 2002; Stokkan et al., 2001; Whitmore et al., 2000).

1.3. Insect clocks

While most circadian systems consist of positive and negative molecular elements the contributing gene set (Bell-Pedersen et al., 2005) as well as the location of the clock in the brain appears to (Helfrich-Förster, 2004, 2005a) differ between species. For example in insect clocks two families of cryptochromes (cry) evolved through several gene duplications and losses and they define three different insect systems (Sandrelli et al., 2008; Yuan et al., 2007). In the first system (*Drosophila* type) CRY protein insect type 1 (CRY1) functions as a photoreceptor in the central brain or as both photoreceptor and central clock gene in peripheral tissues. The second system, found mainly in Lepidoptera, is defined by the presence of both CRY1 and CRY2 (the latter is not light sensitive) and represents the ancestral system. In the third clock system only CRY2 (also called mammalian type

CRY) is present, but its function in the core clock seems to be modulated differently in beetles and bees, because of a different set of interaction partners (*A. mellifera* clock does not have the *timeless* gene). There are similarities and differences between insect clocks concerning the location of clock neurons in the brain. In most insects cell bodies of the clock neurons are located in the lateral and dorsal regions of the brain and can be subdivided into different clusters (Tomioka and Matsumoto, 2010). In crickets, locusts, cockroaches, phasmid and cicada there are additional neuron clusters in the lamina of the optic lobe (Abdelsalam et al., 2008; Homberg et al., 1991a; Sato et al., 2002; Wei et al., 2010). Experiments in which the lamina of crickets was ablated point to some, but a minor, function of the lamina neurons in circadian control of locomotion (Abdelsalam et al., 2008; Okamoto et al., 2001).

1.4. The circadian clock of bees

First evidence for a circadian clock in bees was given by the description of diurnal foraging behavior of honey bees and their remarkable time memory (Beling, 1929), which enables them to synchronize their flower visits to the most rewarding times of the day (Kleber, 1935). Honey bees have further been shown to use their circadian clock for time compensated sky compass orientation during flight (Beier and Lindauer, 1970) and their famous “waggle dance” communication (von Frisch, 1965).
So far, few studies concentrated on the circadian behavior of bees and little is known about the molecular mechanism and the neuronal organization of the bee clock. This is especially true for solitary bees. Bees (Hymneoptera: Apoidea: Anthophila) as pollinators are an important part of ecological systems and are of particular agricultural and economic value to us. Adapted to diurnal floral resource availability, bees forage throughout the day whereby foraging maxima vary with temperature and weather conditions and differ between bee species creating foraging niches for the different bees (Gottlieb et al., 2005; Vicens and Bosch, 2000).

Life history and rhythms of bees

Both bee types introduced in this thesis are generalist pollinators native to Europe but they adapted a different life style to cope with environmental changes. While the mason bee (Apoidea: Megachilidae; two species investigated in this thesis: *Osmia bicornis* (old name: *O. rufa)* and *Osmia cornuta*) lives solitary, the honey bee *Apis mellifera* (Apoidea: Apidae), is in a hive community with thousands of other individuals.

Honey bees

The honey bee eusocial system is based on two female casts, queen and worker, whereby only the queen reproduces with the male bees (drones) (Winston, 1987). After returning from the mating flight the queen stays in the hive for egg laying and sterile worker bees fulfill all other tasks necessary to keep the hive community alive (Free, 1964). Thereby task allocation in honey bees is determined by

age dependent polyethism with genotypic variability (Calderone and Page, 1988) and transitions between tasks are accompanied by behavioral and physiological changes (Elekonich and Roberts, 2005; Winston, 1987). At the age of 2-7 days adult worker bees carry out intensive brood care, but they do so without displaying any rhythms in behavior, while older bees performing tasks at the periphery or outside of the hive, especially foragers, are highly rhythmic (Moore et al., 1998; Seeley, 1982) (**Fig. 2**).

Young nurse bees are behaviorally arrhythmic, which enables them to take care of the brood around the clock and thereby optimize colony development. In line with this hypothesis no rhythms in queen and court behavior or oviposition were detected so far (Free et al., 1992; Harano et al., 2007; Johnson et al., 2010). Nevertheless, this behavior is highly plastic and task related. For example, in case of a shortage of foragers, nursing bees switch to foraging with diurnal activity rhythms, while foragers can go back to attending the brood without any rhythms if there are not enough young nursing bees in the colony (Bloch and Robinson, 2001). The same plastic behavior is true for queens, which show rhythmic behavior only when they are not mated or outside of the colony environment when they are not laying eggs (Harano et al., 2007). Eban-Rothshild and co-authors argue that plasticity in bee behavior is ancient and not related to the evolution of caste system because the primitively eusocial bumble bee queens are arrhythmic while attending their first brood batch in spring (Eban-Rothschild et al., 2011). The elements modulating behavioral rhythmicity are unknown, but Juvenile hormone (JH) could be involved in regulating plasticity in rhythmic behavior, because increasing JH titers mediate task transition from nursing to foraging (Robinson, 1987). Unfortunately no causal connection between changes in JH titers and the arrhythmic behavior of nurses could be drawn (Bloch et al., 2002), although JH titers show diurnal cycling (Elekonich et al., 2001). It is also possible that hormones released by the brood might modulate rhythmicity in behavior of nurses, because switches in rhythmicity depend firstly on the colony environment, but also direct physical contact to the brood

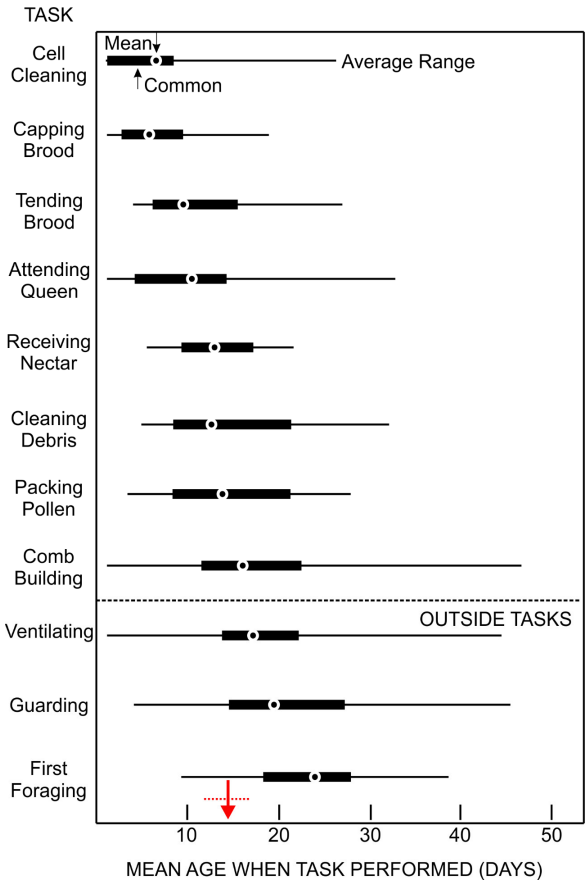

Figure 2: Life history and age dependent task transition in honey bees.
Bees show a typical onset of rhythmicity in behavior (red arrow) at the age of 2 weeks (coinciding with the transition to outside tasks), whereby the exact time point varies between individuals and genotype (slow or fast transition genotype) and is task related. (Figure based on Winston, 1987)

seems to be necessary. Indeed, nurses unable to perform their task because caged on a brood less comb inside the hive become rhythmic shortly after separation (Shemesh et al., 2010). Nevertheless recently it has been shown that brood pheromones when applied orally as larvae extract or synthetic brood ester pheromone do not cause arrhythmic behavior of nurse bees, but curiously presence of capped brood does (Nagari et al., 2017a).

Another extraordinary phenomenon of the social honey bee clock is its ability of social synchronization that was elegantly demonstrated by Frisch and Koeniger (Frisch and Koeniger, 1994) but also shown in earlier studies (Medugorac and Lindauer, 1967; Moritz and Sakofski, 1991). Light dark (LD) cycles can entrain the bee clock (Bloch et al., 2001; Frisch and Koeniger, 1994; Ludin et al., 2012). But the clock responds also to many other environmental time cues such as temperature and humidity (Fuchikawa and Shimizu, 2007a). Further manipulations like anesthesia (Cheeseman et al., 2012; Medugorac and Lindauer, 1967) or imposed feeding cycles (Frisch and Aschoff, 1987; Simone-Finstrom et al., 2014) are able to reset the bee clock, too. Unlike solitary insects, honey bees live in colonies, consisting of typically several thousand individuals, which requires synchronization of activities at various levels: Worker bees synchronize their different tasks in the beehive, e.g. receiving nectar from the foraging nest mates as well as donating food to them (Crailsheim et al., 1996, 1999). Foragers are concertedly directed to floral resources (“social foraging”) (Seeley, 1986) and also queen bees and drones have to synchronize their mating flights to define times of the day (Koeniger et al., 1996). Analogously to the circadian rhythms of single bees, the collective rhythmic activity of bees in a colony can be shifted by light, temperature, feeding cycles and social Zeitgebers (Frisch and Aschoff, 1987; Frisch and Koeniger, 1994; Kefuss and Nye, 1970; Moore and Rankin, 1993; Moritz and Kryger, 1994). It was discovered that both worker and queen bees can function as social Zeitgebers (Moritz and Sakofski, 1991; Southwick and Moritz, 1987) and shift the activity rhythm in the hive. For young bees the social Zeitgeber seems to be most important and overrides the light entrainment (Fuchikawa et al., 2016), but the define cue of social synchronization remains unknown.

Queen, worker and drone do not only differ in their function in the hive community but also in their development. Sex is determined genetically (*complementary sex determiner* gene) and embryos in unfertilized (haploid) eggs develop into drones in the course of 24 days (Bertholf, 1925; Beye et al., 2003). Female worker bees hatch from fertilized eggs, live through 5 larval instars, two pre-pupal and 9 pupal stages and emerge from their brood cells 21 days after oviposition (Bertholf, 1925; Groh and Rössler, 2008; Hendriksma et al., 2011). By adjusting the larval diet female castes are determined via differential gene expression mediated by DNA methylation (Kucharski et al., 2008; Lyko et al., 2010), whereby pre-adult development of queen bees is shorter (16 days) (Bertholf, 1925). Colony environment is also able to modulate the development of the circadian system. Young bees deprived from their social context are significantly later able to express rhythmic behavior (Eban-Rothschild et al., 2012; Meshi and Bloch, 2007). Social interaction of nurses with foragers can further inhibit task related maturation of their hypopharyngeal gland, which switches from producing larval food to nectar processing enzymes with the transition to foraging (Meshi and Bloch, 2007). This demonstrates the importance of including the social context into experimental design when investigating honey bee behavior.

Solitary bees

In phylogenetic trees Apidea and Megachilidae cluster together as sister families based on morphology (highly modified first and second labial palpal ("long tounged bees")) and gene sequence analysis (Hedtke et al., 2013). Advanced sociality evolved only in the Apidae family, but there are also some cases of primitive eusocial behavior among species of the other 5 bee families (Cardinal and Danforth, 2011; Winston, 1987). The Megachilidae are typical solitary bees and overwinter in diapause, whereby dormancy stage in winter varies with the species. For example *Megachile rotundata* (Megachilidae) endures the winter as prepupae, while most *Osmia* species (Megachilidae) arrest in diapause as adult bee (James and Pitts-Singer, 2008). Adult dormancy is a derived trait among solitary bees and enables them to emerge earlier in spring (Bosch et al., 2001). *O. bicornis* (red mason bee) typically emerges in March and April and *O. cornuta* a few weeks earlier with variations between populations based on phenological differences (James and Pitts-Singer, 2008; Krunić and Stanisavljević, 2006) (**Fig. 3**).

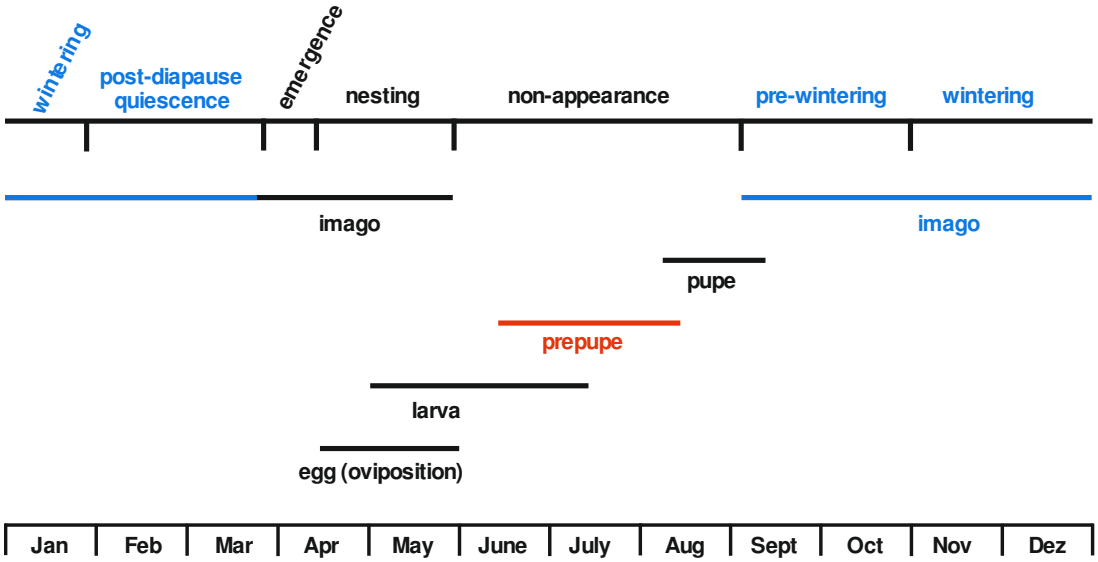

Figure 3: Life history of solitary mason bees (*O. bicornis*).
In spring the bees emerge, mate and pollinate during nesting season. Development of offspring from different populations are synchronized to winter onset by the prepupal summer diapause (red) which is terminated in late summer/ early autumn. Imagos overwinter in a three stage dormancy (blue): pre-wintering, wintering, post-diapause quiescence. (Figure based on Wasielewski et al., 2011a)

Emergence is triggered by temperature raise in spring. Males emerge 2-4 days earlier than females and wait at the nest site until they can mate with the freshly emerged females (Bosch and Blas, 1994; James and Pitts-Singer, 2008). This time difference is probably caused by sex specific temperature thresholds. After mating the female mason bees build a nest in tube shaped cavities, like hollow plant, by creating a row of brood cells with mud and plant material and each brood cell is equipped with an egg and pollen provisions, lining up females first and males last (Bosch et al., 2001; Wilkaniec and Giejdasz, 2003). In these cells the eggs develop through five larval instars (Rust et al., 1989) and enter a summer diapause in the prepupal stage (Bosch and Vicens, 2002; Kemp et al., 2004) with diapause termination dependent on temperature regime, whereby development of offspring from different populations is synchronized to winter onset (Sgolastra et al., 2012). In late summer the imago

instar appears and overwinters immobile inside a cocoon until increasing temperatures in spring induce the JH dependent diapause termination (Wasielewski et al., 2011b). The reproductive diapause in *Osmia* is characterized by continuation of ovary development and energy reserve depletion (Kemp et al., 2004; Wasielewski et al., 2011a, 2013). Winter dormancy in *Osmia* can be separated into three different stages: pre-wintering stage (eclosed bees prepare for diapause), wintering (diapause corresponding phase), post-diapause quiescence (dormant bee awaits temperature rise in spring) (Sgolastra et al., 2010; Wasielewski et al., 2011a, 2013). Only in the wintering stage ovary development and midgut/crop metabolic activity is slowed down significantly (Wasielewski et al., 2011a, 2013). Further oxygen consumption drops at diapause onset and gradually increases until a plateau in the post-diapause quiescence (Sgolastra et al., 2010). In contrast to pre-pupal diapause, adult diapause in *Osmia* is obligate. Moreover the latter is fitness cost associated because of continuous metabolic activity and development during the dormant months (Bosch et al., 2010).

The solitary bee has been widely used for research on annual timing, but circadian rhythms remain largely unattended in this model. The solitary bee's complex seasonal life cycle demands elaborated experimental design. Emergence can be delayed by keeping the bees at low temperatures, but metabolic processes in the dormant bee continue and resulting effects on development and decreased life expectancy of these bees have to be considered in study design (Bosch and Kemp, 2003, 2004, Dmochowska et al., 2012, 2013). So far, a few studies on circadian emergence rhythms and oxygen consumption rhythms in *M. rotundata*, a prepupae wintering species, were performed with the aim to determine optimal storage conditions for artificial raising by beekeepers. Tweedy and Stephen could identify temperature as a major entrainment factor for emergence rhythms and oxygen consumption in adults, while light pulses entrained neither of these outputs regardless of the developmental stage (Tweedy and Stephen, 1970, 1971). The circadian system of *M. rotundata* is highly sensitive with temperature pulses of 2 °C (or less) sufficient to synchronize emergence to temperature rise (Yocum et al., 2016). Further there might be a temperature amplitude dependent mechanisms to synchronize emergence of bees nesting in caverns of different depths (Yocum et al., 2016).

Components of the bee clock

On the transcriptional level, several candidate genes for the bee clock have been described. Rubin and co-authors showed circadian mRNA cycling in brains of honey bees. According to the model for the bee molecular clock based on these data, *period* (*per*), *mammalian type cryptochrome* (*cry2* or *m-cry*), *clock* (*clk*) and *cycle* (*cyc*) contribute to a negative TTFL (Bloch, 2010; Rubin et al., 2006; Weinstock et al., 2006). The proteins PER and CRY2 form a heterodimer, they are translocated into the nucleus and prevent the formation of the CLK:CYC complex, which inhibits transcription of *per* and *cry2* (**Fig. 4**). While transcript levels of *per* and *cry2* oscillate in the same phase with highest levels at midnight, the peak of *cyc* expression is delayed by approximately 6 hours and *clk* expression does not oscillate at all. Further there are orthologs to the *Drosophila* clock genes *par domain protein 1* (*pdp1*) and *vrille* (*vri*) in the honey bee genome and *clk* and *cyc* encode putative binding sites for PDP1 and VRI. The orthologs of these two proteins in *D. melanogaster* influence the first negative feedback loop in a second positively regulating feedback loop (Cyran et al., 2003). There are no orthologs to the central clock gene *timeless* (*tim1*) in *Drosophila* or the insect type *cryptochrome* (*dcry or cry1*), encoding a blue-light photoreceptor involved in the light input pathway in *Drosophila*. But there are also other cycling genes which might contribute to the clock or be influenced by it, like for example *timeout* (*tim2*) and *clockwork orange* (*cwo*), which are involved in light entrainment and transcriptional regulation of clock genes in the *Drosophila* clock (Benna et al., 2010; Kadener et al., 2007; Richier et al., 2008), or the visual system associated gene *arrestin-2* (Rodriguez-Zas et al., 2012; Rubin et al., 2006).

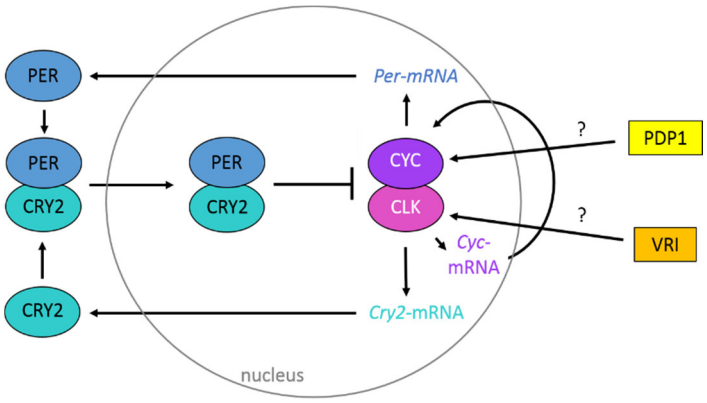

Figure 4: Working model for the molecular clock mechanism in the honey bee.
The negative elements PER and CRY2 regulate their own transcription via inhibition of the CYC:CLK complex (positive elements) in a negative feedback loop. In another feedback loop cyc but not clk expression oscillates and PDP1 and VRI might be involved acting as positively regulating transcription factors similar to the mechanism in *D. melanogaster*. (Figure based on Bloch 2010)

While the molecular clockwork of the honey bee is not yet fully understood, there are indications that the task related plasticity in behavioral rhythms is associated with modifications on the molecular level (Shemesh et al., 2007, 2010; Toma et al., 2000). Real time quantitative polymerase chain reaction (qPCR) experiments showed that the mRNA levels of clock genes *per*, *cyc*, *cry2* and *tim2* in honey bee brains oscillated in a daily manner significantly in foragers, but not, or at rather low amplitude, in nurses (Shemesh et al., 2007, 2010). This was the case regardless of the light treatment, flight experience or colony type, but depending on the performed task (Bloch et al., 2004; Shemesh et al., 2010). Furthermore, in slot blot and Northern blot experiments elevated levels of *per* mRNA were detected in forager brains, consistently with task assignment in premature forager bees and not simply an effect of ageing (Toma et al., 2000). In further experiments, in which age and task performance

were uncoupled, the difference in daily oscillation patterns in *per* mRNA between foragers and nurses of different ages was successfully connected to the task (Bloch et al., 2001). In addition, the regulation of overall *per* mRNA levels may be developmentally regulated, because in general older bees had higher levels of *per* mRNA (Bloch et al., 2001, 2004). Nevertheless, this does not mean that the clock in nurses is not functional. Nurse bees removed from the hive soon start to exhibit circadian activity under constant conditions with a phase fitting to the ambient day-night cycle (Shemesh et al., 2010).

The localization of the clock in the bee brain and PER Protein

In order to fully understand the function of the bee clock, experiments on the protein level and the neuroanatomical characterization of clock neurons in the bee brain are required. The PER protein, as part of the central TTFL, is a self-evident target to perform neuroanatomical studies on. In several insect species, pacemaker neurons have been identified as cell clusters in the dorsal and lateral brain (e.g. in fruit flies (Helfrich-Förster, 2005b; Helfrich-Förster et al., 2007a; Siwicki et al., 1988), hawkmoth (Wise et al., 2002) or beetles (Frisch et al., 1996)), or only in the dorsal brain (e.g. in silkmoths (Sauman and Reppert, 1996; Sehadová et al., 2004)). In the honey bee one study describes *per* expressing neurons in the dorsal protocerebrum, between medial and lateral calyces of the mushroom bodies, one anterior and one posterior situated cluster (Bloch et al., 2003). Furthermore, several PER-positive cells were detected in the optic and antennal lobes. While these showed *per* expression in the nucleus, the cells in the protocerebrum had exclusively cytoplasmic PER-positive staining. Number of PER-positive cells and staining intensity oscillated during the day. There was a significant difference in the comparison of cell number of older (foraging) bees and younger (hive) bees.

Neuropeptide pigment dispersing factor (PDF)

Besides core clock proteins involved in the TTFL, another factor was shown to essentially contribute to the normal circadian time keeping in several insects such as cockroach, cricket, beetle, and fly: the neuropeptide Pigment Dispersing Factor (PDF) (Helfrich-förster et al., 1998). In surgery experiments severing or ablating PDF neurons (Shiga and Numata, 2009; Stengl and Homberg, 1994) disrupted rhythms in locomotor activity while transplantation of the PDF innervated brain area restored the clock function (Reischig and Stengl, 2003a). Further studies with fly mutants confirmed the central role of PDF in the circadian clock (Helfrich-Förster, 1998; Renn et al., 1999). PDF injections into the accessory medulla, a small neuropil with very high PDF cell density, caused shifts in the activity rhythms of cockroaches and crickets (Petri and Stengl, 1997; Singaravel et al., 2003). In Drosophila, circadian cycling in PDF staining intensity and daily changes in morphology of dorsal PDF axon terminals have been demonstrated (Fernández et al., 2008; Park et al., 2000) and interpreted as oscillations in peptide release. Also in honey bees circadian cycling of *pdf* expression, at least on the transcriptional level, has been shown (Sumiyoshi et al., 2011). PDF is therefore regarded as output factor. Furthermore, at least in *Drosophila*, PDF seems to be a communication element between the different clock cell clusters (Lin et al., 2004), because most of the clock neurons have PDF receptors (Im and Taghert, 2010). In honey bees, there is another putative function of PDF. Zeller and co-authors observed the PDF arborization network in the optic lobe overlapping with elements of the polarized light input pathway and speculated on its potential role in integrating the circadian clock into time compensated sky compass navigation (Zeller et al., 2015).

PDF expression patterns in insects have been characterized first in orthopterid insects such as locusts, crickets, and cockroaches, and to some degree in lepidoptera (Homberg et al., 1991a, 1991b). Very detailed neuroanatomical descriptions are available for the cockroach and the fruit fly (Helfrich-Förster, 2005b; Helfrich-Förster et al., 2007b; Stengl and Homberg, 1994; Wei et al., 2010), but there are many other insects in which PDF neurons have been described so far (Nässel et al., 1991, 1993; Sauman and Reppert, 1996; Sehadová et al., 2003; Závodská et al., 2003, 2009). In most insects, there is a group of PDF somata positioned in the lateral brain adjacent to the proximal side of the medulla. In some species there are additional PDF cell clusters in the optic lobe (f.e. mayfly and some locust species) and in the central brain (f.e. mayfly, waterstrider and some lepidoptera) (Sauman and Reppert, 1996; Závodská et al., 2003). In other lepidopteran species PDF somata seem to be exclusively located in the dorsal brain (Homberg et al., 1991b). PDF cell localization in bee brains has been characterized in several studies with different antibodies (Bloch et al., 2003; Sumiyoshi et al., 2011; Weiss et al., 2009). The cell bodies of PDF neurons cluster together in numbers of 9-20 in the lateral protocerebrum and arborize in a complex fiber network throughout the brain. Bloch and co-authors did not detect co-expression of *per* and *pdf*, which is also the case in some lepidopteran species (Sauman and Reppert, 1996; Sehadová et al., 2004; Wise et al., 2002). The high complexity and the possible multiple functions of PDF in circadian time keeping demand a more detailed neuroanatomical description combined with co-expression studies, time course experiments and manipulation experiments to assess the role of PDF in the bee clock.

1.5. Aim

The eusocial honey bee and the solitary bee adapted different mechanisms in their communal and reproductive life style. While it has been shown that the social lifestyle of the honey bee has an effect on the circadian rhythm in behavior of nursing bees it is unknown what mechanistic modifications to the circadian clock have evolved along with sociality in bees. By investigating the circadian clock of two bee types, one highly social and the other solitary, we achieve a better understanding of effects of life style on the endogenous clock and the functional consequences. Currently genetic manipulations are not well established in the social hymenoptera field. Therefore, I followed a comparative approach of evolutionary developmental studies and combined investigations on the clock across different bee species and different developmental stages. For characterizing the localization of the circadian clock in the bee brain I conducted immuno-fluorescent staining of the clock component PER, for which I used a new antibody, which had to be tested for specificity. For the other clock component, PDF, I used the anti-PDH (Pigment Dispersing Hormone) antibody, which has been shown well suited for cross species studies. Furthermore, I needed to establish a method to monitor individual circadian output behavior of the different bee types, with or without the social context of a honey bee colony, to better understand the effect of sociality on circadian behavior. Additionally, I harnessed the locomotor activity set up for monitoring emergence rhythms in solitary bees, which overwinter in adult stage, because there is no report of circadian emergence rhythms in honey bees and only one other solitary bee species (diapausing as pre-pupae) so far. I aimed to compare the clock of social and solitary bee with respect to behavioral output, neuroanatomy, but also in clock development, which may be different due to the different life history of the two bee types. Thereby, I aim to answer the question whether and which modifications to the circadian system evolved along with different social life styles.

2. Material and Methods

2.1. Material

2.1.1. Insects

2.1.1.1. Honey bees

The honey bees used in this thesis are of the species *Apis mellifera*, subspecies *carnica* and the colonies were kept at the University of Wuerzburg, Department of Animal Ecology and Tropical Biology. Queens were inseminated by multiple drones. Standard beekeeping methods were applied with bees kept in field colonies housed in three-story Langstroth standard hives. These colonies consisted of approximately 35 000–40 000 bees and the two lower supers contained the brood nest, which spanned over six to seven frames.

2.1.1.2. Solitary bees

Cocoons of solitary bees, *Osmia bicornis* and *Osmia cornuta*, were purchased at a commercial beekeeper (WAB-Mauerbienenzucht, Konstanz) and stored at 4°C and 60% RH until the start of the experiments. This allows to perform experiments from March to August since the natural emergence could be postponed for several months in case of *Osmia bicornis* and for some weeks in case of *Osmia cornuta*.

2.1.1.3. Flies

Two strains of *Drosophila melanogaster* from the stock collection of the University of Wuerzburg, Neurobiology and Genetics, raised on *Drosophila* standard media in fly vials (5 cm^2 x 8 cm), were used in this thesis. One was the wildtype strain Canton-S and the other loss of function mutation of *period* mutant per^{01} (Konopka and Benzer, 1971).

2.1.2. Antibodies

Antibodies for immunocytochemistry are listed in **table 1** together with additional information concerning their immunogen, donor animal, working concentration and source. The antibodies were stored containing NaN_3 (0.02%) at -20°C in glycerol (1:1) or in small aliquots at 4°C. The dilutions (in 5% NGS-PBST (0.5%)) and 0.02% NaN_3) were kept at 4°C.

Table 1: Antibodies used in this thesis.
Invitrogen/ThermoFisher Scientific Inc. Waltham MA, USA

Primary antibodies	**Immunogen**	**Donor animal**	**Working concentration**	**Source/Reference**
Anti-β-Pigment dispersing hormone	*Uca pugilator* β-PDH conjugated to bovine thyroglobulin (NSELINSILGLPK VMNDA-NH_2)	rabbit	1:3000	H. Dircksen (Dircksen et al., 1987)
Anti-*am*Period	*am*PER	rabbit	1:100 (*Apis, Drosophila*), 1:500 (*Osmia*), 1:1000 (*Apis, Osmia*)	Eva Winnebeck (Fuchikawa et al., 2017)
Anti-Synapsin 3c11	SYN	mouse	1:50	Würzburg stocks (Klagges et al., 1996)
Anti-Horseradish peroxidase	Horseradish peroxidase (in insect central nervous system a group of membrane protein epitopes called Nervana)	rabbit, conjugated to Cy™3 AffiniPure	1:300	Jackson ImmunoResearch
Secondary antibodies	**Immunogen**	**Donor animal**	**Dilution**	**Source/Reference**
Alexa Fluor 488	anti-rabbit anti-mouse	goat	1:200	Molecular Probes (Invitrogen)
Alexa Fluor 555	anti-rabbit			
Alexa Fluor 635	anti-rabbit			

2.2. Methods

In this section I will explain the general procedures that I followed in my experiments. I collected bees and entrained them (or they were entrained by the natural daylight cycle) in the laboratory. At certain time points of the day I sampled them for immunofluorescent staining experiments or I introduced the bees into the locomotor activity set up for monitoring behavior. Several details, like sample size and sampling time points that varied between experiments will be specified in the corresponding results section. I sub-classified the following two sections ("Collection and entrainment" and "Locomotor activity") into experiments performed with honey bees or with solitary bees. The section "Immuno fluorescence" is described in general, but it is stated in the text which protocol I used for which species.

2.2.1. Collection and entrainment

Honey bees

Free flying honey bees were caught with a custom-made aspirator (**Fig. 5 A**) and were housed in small groups of ca. 15-30 bees in one plastic cage (12 cm^2 x 8 cm) (adapted Polystyrol fly rearing vials, T-TK e.K., Retzstadt, Germany) (**Fig. 5 B**). The bees were provided with Apiinvert (Südzucker AG, Mannheim, Germany) sugar syrup and a piece of bees wax. The cages were placed in a climate chamber (20°C) until sampling for immunofluorescent staining with illumination switching to DD at the time of natural sunset. Time of sunset differed between experiments and is indicated in the results section (together with the sampling time point).

Solitary bees

Cocoons of solitary bees, if not used directly in emergence behavior experiments, were placed into an emergence box (15.5 cm x 10 cm x 6 cm) in a flight tent (**Fig. 5 C**/**D**) for one day at 10°C and 45% RH to adjust to the temperature rise and then they emerged at 18°C and 60% RH. After emergence I dot marked them with paint (Uni posca, Mitsubishi Pencil Co., Tokyo Japan) on the thorax and released them into a flight tent. This was equipped with bundles of transparent micropipette tips (1000 µl, Finntip 1000, Thermo Labsystems, Vantaa, Finland) to provide the bees a hiding and sleeping place (**Fig. 5 D**).

Entrainment

Honey bees

Honey bees caught from the hive for PER and PDF immunofluorescence staining in adults were entrained to the prevailing natural conditions in the hive, which has been housed outside. They were collected in the afternoon and one group sampled right away. Another group was placed in a plastic

cage (12 cm^2 x 8 cm) with sugar syrup in an incubator (20°C, 60% RH) and light switching off at the time of sunset until sampling in the following night. Light intensity during light phase was 200 lux. The ambient LD regime varied between experiments due to strong variation in daylight time during the year.

Solitary bees

Osmia were not naturally entrained and kept either in small groups (approx. 20 individuals) in plastic cages (12 cm^2 x 8 cm) (for PER and PDF double staining) or in big groups (approx. 100 individuals) in the flight tent (for staining of different developmental stages/ages of adult *O. bicornis*). In general, entrainment conditions in the lab have been applied to the test subjects for 3-5 days before they were sampled. Light intensity during light entrainment (LD 12:12) in plastic cages (for PER and PDF characterization in adults) was 200 lux, in the flight tent it was slightly lower (100 lux). In the flight tent, bees received additionally temperature entrainment (for experiments with different developmental stages in *O. bicornis*). The temperature amplitude between “day phase” (12 h) and “night phase” (12 h) was 7°C (25°C/18°C) for staining experiments. Temperature entrainment in behavioral experiments is stated at the corresponding section (2.2.2 Locomotor activity monitoring).

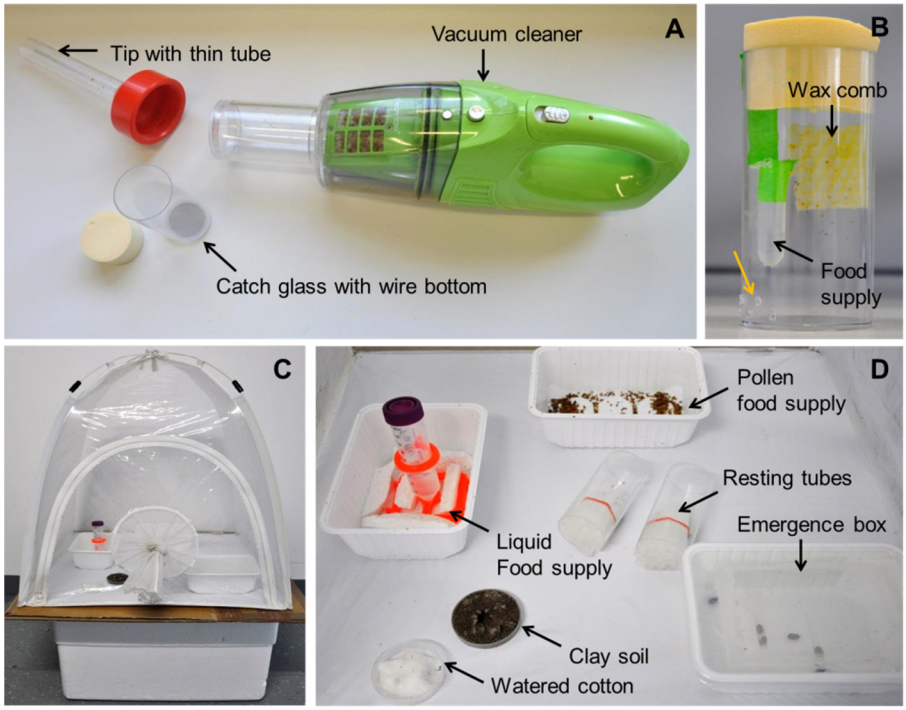

Figure 5: Equipment for bee collection and entrainment housing.
A: custom made aspirator consisting of a modified vacuum cleaner with a thin tip to aspirate single bees. Between tip and vacuum cleaner a catch glass (plastic glass, adapted fly rearing vial) with a wire grid on the bottom is inserted, to prevent bees from being sucked into the vacuum cleaner. The catch glass can be closed with a foam plug. **B:** Entrainment housing for immunofluorescence experiments made of the same fly glasses by inserting a piece of bees wax, liquid food (provided in a microcentrifuge tube with a hole in the bottom) and air ventilation holes (yellow arrow). **C:** Flight tent, which can be accessed to collect bees by a small opening with gaze surrounding (round opening in the front). Two sides are made of plastic foil, while the other two sides (not seen in the picture) are made of gaze to ensure accurate ventilation. **D:** Inside of the flight tent is equipped for housing solitary bees. They are provided with two different food sources (liquid: Apiinvert sugar syrup, pollen food supply: honey bee pollen pellets) an extra water source (watered cotton) some clay soil for nest building and bundles of 1000 µl micropipette tips they can use for resting and nesting (resting tubes). The emergence box has a transparent lid with a piece of gaze inserted for ventilation.

2.2.2. Locomotor activity monitoring

2.2.2.1. Monitoring activity of honey bees

I monitored locomotor activity of individual honey bees in the social colony context under controlled laboratory conditions. For this purpose I connected a commercially available red-light barrier (IR) based locomotor activity monitoring system by TriKinetics (TriKinetics Inc. Waltham, MA USA) with a single frame mini hive (Fig. 6).

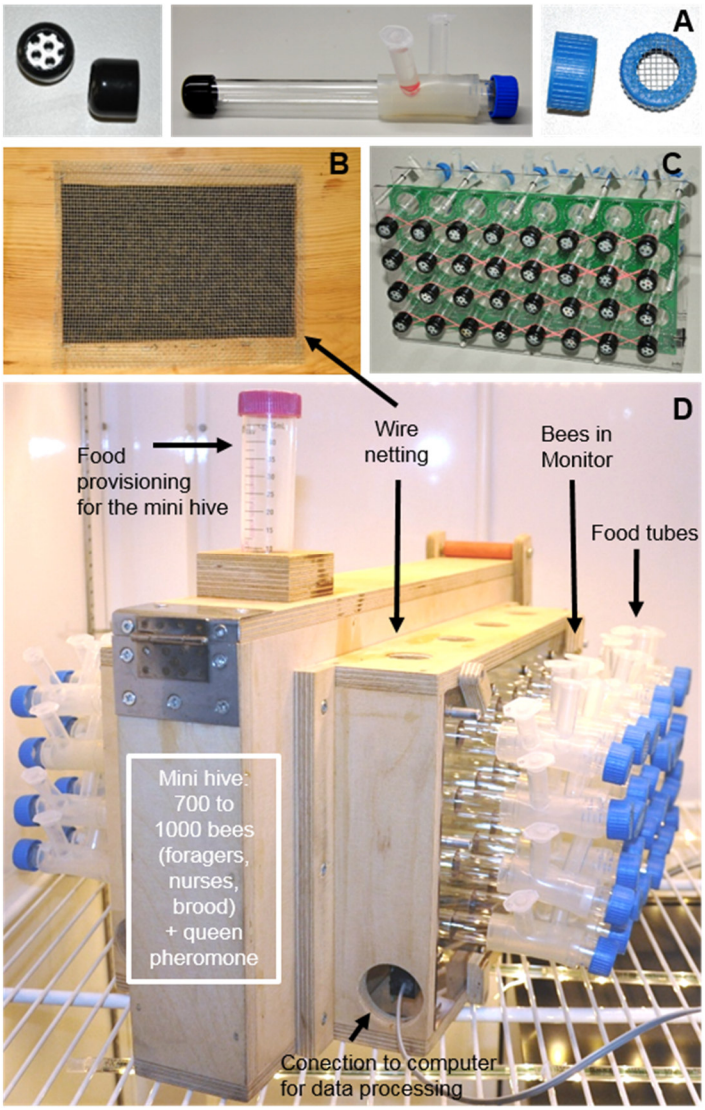

Figure 6: Photographs of the activity monitoring set-up in social context.
A: monitor glass tube with ventilation caps on both endings and individual food supply. **B:** wooden box containing a mini colony with wire netting on the sides. **C:** 32 glass tubes with bees placed in the locomotor activity monitor. **D**: complete set-up: monitors are connected to a computer interface (black arrow). (Adapted from (Beer et al., 2016))

The mini hive contained a brood comb (open and closed brood) with hive bees and forager bees on it (700-1000 bees (estimation according to (Gerig, 1983)) and Queen pheromone (Bee Boost with queen

mandibular pheromone)) to substitute a real queen to reduce stress caused by removing the bees from the hive and their queen. Bees in the monitors (adapted TriKinetics LAM16 (Locomotor Activity Monitors), Pyrex Glass tubes, 100 mm length, diameter 16 mm) were placed next to the wire netting of the mini hive. They were separated from it by a double mesh system consisting of a ventilation cap at the end of the monitoring tube (**Fig. 6 A**) and the wire netting (**Fig. 6 B**), which allowed them to have contact with the mini colony over pheromones and microclimate (e.g. temperature, humidity, CO_2) as well as vision and vibration, but no direct tactile contact. Control subjects socially isolated from the mini colony (bees in monitors 0.5 m away from the mini hive) were placed in the same incubator. I monitored the activity of the mini colony indirectly by measuring the temperature rhythms in five different places: sensors were placed close to the brood cells, close to the food storing cells and into a tube in the monitor next to the mini hive. As controls, further sensors were placed into a tube in the monitor 0.5 m away from the mini hive and separately in the incubator.

To control for age, newly emerged honey bees were marked and immediately put back into the outdoor hive during one afternoon. 13 days later the marked subjects together with newly emerged bees (< 6h old) were placed into the monitor tubes. In the experiments, these groups are referred to as forager-aged bees and nurse-aged bees. The food supply for individually monitored bees consisted of 2 different diets, both *ad libitum*: a) sugar syrup by Apiinvert (Südzucker AG, Mannheim, Germany) and b) a mixture of pestled pollen pellets and Apiinvert sugar syrup (1:3). This way, the animals could choose the amount of pollen diet suitable for their age. The mini hive had pollen and honey stored in the comb cells around the brood and an extra food supply of Apiinvert sugar syrup, which served as foraging resource and was refilled every 3rd to 4th day in irregular intervals in order to prevent entrainment by feeding cycles. While refilling the additional food tube for the hive, a 3°C temperature drop occurred, but constant temperature was restored in less than 5 minutes. Temperature and humidity conditions in the incubator (Percival INTELLUS, CLF Plant Climatics GmbH, Wertingen, Germany) were otherwise constant (two different trials: 30 ± 0.5°C or 28 ± 0.5°C 50% ± 10% RH (relative humidity)). The illumination of monitoring tubes during the artificial light phase was between 380 and 500 lux, depending on the position of the tube. Light intensity inside the mini hive box directly at the double mesh to the monitoring tubes was 7 – 8 lux.

In a survival analysis the last beam cross of a bee was defined as approximate time of death, because in earlier observations the bees had died in the course of a few hours after their last registered movement.

2.2.2.2. Monitoring activity of solitary bees

Locomotor activity of adult bees

All locomotor activity experiments with solitary bees were conducted with *O. bicornis*. I measured the activity of these with the same IR-beam based monitoring system (LAM16) by Trikinetics without the mini hive (**Fig. 6 A/C**). The food supply consisted of Apiinvert sugar syrup and water *ad libitum*. In preliminary experiments on the investigation of light entrainment of locomotor activity rhythms in adult animals I synchronized the animals in group cages (plastic cage (12 cm^2 x 8 cm)) with a LD-cycle of 12:12 hours (light intensity: 200 lux, light phase was shifted approximately 10 hours to the natural light cycle) for 3 days. Afterwards I put the bees into the monitoring tubes and monitored their activity under DD conditions (20 ± 0.5°C). In a second experiment, I introduced solitary bees into the monitor setup after they had been raised in DD conditions for 10 days (constantly 20 ± 0.5°C, RH 45 ± 10 %). They were then exposed to an LD cycle (12:12, lux during light phase: 200-400 lux) for 3 days and afterwards to DD conditions for 7 days. The following 3 days they were in a LD cycle (12:12, lux during light phase 200-400 lux), which was 6 hours shifted to the previous one. Afterwards they experienced DD conditions again.

Emergence rhythms

To monitor circadian rhythms in emergence I inserted the cocoons, which have been stored at 4°C into individual tubes and synchronized their emergence time by temperature cycles. In these cycles, temperature in the incubator (Percival INTELLUS, CLF Plant Climatics GmbH, Wertingen, Germany) was raised stepwise to the emergence temperature (**Fig. 7**). After four temperature cycles (12:12, meaning 12 hours high temperature and 12 hours low temperature) the environmental conditions were kept constant (19.2°C ± 0.1°C, 75 ± 10% RH, DD) and the first beam crossing displayed the first foraging for food right after emergence. Another set of experiments in which I measured only the circadian locomotor rhythm after emergence in DD were performed similarly. Here I omitted the stepwise temperature rise with cycling temperature. In these two experiments I inserted the cocoons and raised the temperature to 20°C and 25°C respectively (see chapter 3.4.) before monitoring activity under constant conditions (DD, 20°C ± 0.1 °C, 60 ± 10% RH and DD, 25°C ± 0.1°C, 45 ± 10% RH).

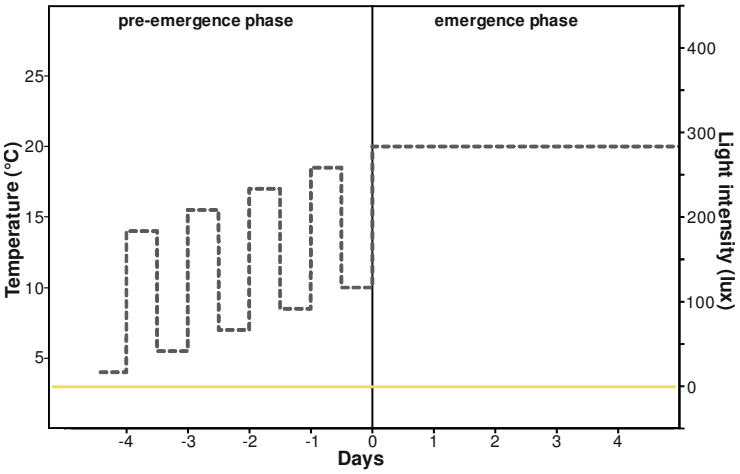

Figure 7: Experimental outline for temperature and illumination during pre-emergence and emergence phase of *O. bicornis*.
During pre-emergence phase temperature (dotted line) is raised in stepwise cycles (from storage temperature: 4°C) to entrain emergence rhythmicity. Emergence phase: constant conditions of 19.2°C and darkness (Light intensity: yellow line).

2.2.2.3. Data analysis

The locomotor activity data was registered in counts (beam crosses) per minute in the Trikinetics system and evaluated with ActogramJ software plugin for ImageJ (Schmid et al., 2011) (Fiji ImageJ Version 1.49, © Wayne Rasband, National Institutes of Health, USA). I determined the endogenous period (τ) and the power of rhythm above significance level for the prevalent period with a periodogram analysis (Chi-square method, *p* level 0.05, Gaussian-smoothing factor 10) and the acrophase (center of gravity in activity) with the acrophase tool. Temperature and humidity data were collected with sensors from MSR (MSR Electronics GmbH, Seuzach, Switzerland; software version MSR 5.24.02) and LabJack (LabJack Corporation, Lakewood, CO 80227 USA; software version LJLog UD V1.18). Mean days were calculated individually and for the test groups in Mircrosoft Excel (2013 Microsoft Office).

2.2.3. *In vitro* rearing of honey bee larvae

In order to standardize sample taking of different developmental stages of honey bees I conducted *in vitro* rearing of larvae (based on (Aupinel et al., 2005, 2007; Hendriksma et al., 2011)). A queen was caged to an empty brood comb for one day to control for larvae age. The cage prevented the queen from leaving one side of the empty brood comb (and excluded the queen after her release on the next day), but workers could pass freely. Larval diet (see **Tab. 2**) was placed into artificial rearing cells (Cupularve Nicotplast ©, Maisod, France). Rearing cells were placed in 48-well plates equipped with pieces of dental rolls (Celluron, Hartmann) soaked in antimicrobial MBC-glycerol solution. Three days after the queen had been released from the comb cage first instar larvae were grafted into the diet filled rearing cells in the laboratory three days later. The plates were kept at 35°C ± 0.1°C and 97 ± 10

% RH in a rearing chamber (Memmert, loading model 100-800, 91107 Schwabach, Germany) except for feeding which was performed on a heating plate (35°C) and with pre-heated diet (35°C). I fed the larvae daily except for day 2 by pipetting different amounts of larval diet into the rearing cells. Pipetting had to be performed slowly and carefully in order not to drown the larva floating in its food. The diet composition was 50% royal jelly/ sugar solution (w/w) whereby the sugar concentration varied between three different sugar solutions (see diet A, B and C, Appendix 6.5.) and the amount of food increased with age (**Tab. 2**).

Table 2: Timetable for feeding and sampling larval instars during *in vitro* rearing.
Feeding protocol started on day 1 when larvae were grafted into artificial rearing cells. Diet composition and amount changed with age and corresponding larval instars are indicated below.

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Diet	A		B	C	C	C
Amount	20µl		20µl	30µl	40µl	50µl
Instar	L1 – L2	L2 – L3	L3 – L4	L4 – L5	L5	L5

I sampled larval instars L1, L3, L5 (determined by age, see also (Hendriksma et al., 2011)) and different pupal stages P1, P3, P5, P7, P9 (determined by age and morphology according to (Groh and Rössler, 2008)). P1 were white eyed with white body, P3 orange/pink eyed with white body and P5 were red-brown eyed and already showed cuticle tanning of the first two leg pairs and yellow head and thorax. In P7 the bees had light brown bodies with speckled legs and folded grey wings. On the day before emergence bees entered the last pupal stage, P9, and appeared already adult-like with dark grey thorax and unfolded wings, but they were motionless.

Feeding, survival checks and sampling occurred always at the same time of the day (on the subjective morning resembling ZT0) and the bees were otherwise undisturbed in the incubator. This way I avoided potential desynchronization of individual endogenous rhythms.

2.2.4. Immuno-fluorescence in insect brains

Insect brain tissues were stained via immunofluorescence to visualize proteins which are involved in the regulation of the endogenous clock. First, I used a newly generated antibody which was specifically raised against the PER protein in *Apis mellifera* (*am*PER) (kindly provided by Eva Winnebeck). The other clock component antibody was anti-βPDH antibody, raised against the crustacean pigment dispersing hormone, which recognizes the PDF peptide in insects (kindly provided by Heinrich Dircksen (Dircksen et al., 1987)). Further I used antibodies against Synapsin (SYN) and Horseraddish peroxidase (HRP) to visualize the neuropils in the brain and mark neurons specifically. I stained nuclei with DAPI (4', 6-diamidino-2-phenylindole) to specify PER and PDF staining in sub-

compartments (either nucleus or soma of the cell body) in double labeling experiments. Washing and incubation steps were performed in custom-made baskets (shortened micropipette tip with a plastic mesh on the bottom) fitting into the wells of a 48-well plate (adult brains) and a 96-well plate (larva brains). For each step the basket was transferred to a new well. Volume of washing and antibody solution was 1000 µl in case of adult brains and 200 µl in case of larva brains.

2.2.4.1. Staining protocol

The protocols used in this thesis vary with species and antibodies. The general procedure of an indirect method of immunohistochemistry is presented in the protocol for fluorescent staining in whole mounts of bee brains (**Tab. 3**) and variations of this protocol are described in the subsequent sections. For the dissection I used two sharp forceps and an injection needle to open the head capsule of the adult bee. On ice immobilized bees were decapitated and their heads were pinned down in liquid wax, which was heated with a soldering iron. The head capsule was opened by cutting a window into the cuticle of the frontal head with an injection needle. The side incisions of the window were along the compound eye rim, another incision was made across the ocelli and a fourth at the base of the antenna. After fixation all tissue around the brain, was removed and the brain separated from the rest of the head. Then the back of the brain was freed from trachea and tissue, too.

Table 3: Protocol for fluorescent staining in whole mount bee brains of adult and pupal stages. Procedure used in experiments with *A. mellifera* (pupae and adults) and *Osmia*. Procedures of storage and antigen-retrieval step were used for all adult and pupal bee brain preparations and are stated in more detail in the corresponding methods section. The concentration of antibodies is indicated in material (chapter 2.1.2.). I used different concentrations of anti-*am*PER antibody: for experiments in *A. mellifera* concentrations were 1:1000, 1:100 (pre-absorbed), in *O. bicornis* brains 1:1000 (pre-absorbed), and in *O. cornuta* 1:500 (pre-absorbed). For SYN and PDF double staining in experiments on different developmental stages secondary antibodies Alexa Flour 488 and 635 goat anti rabbit were used. In PER single and double staining experiments (see chapter 2.2.4.6.) *am*PER was labeled with Alexa Fluor 488 goat anti rabbit.

Step	Method	
1	**Pre-fixation preparation**	Opening head capsule in PBS by cutting a window into the cuticle of the frontal head with an injection needle
2	**Fixation**	Incubate heads in 4% PFA in PBST (0.1 %) on shaker 3-4h at RT
3	**Washing**	3 x in PBS, each wash 10 mins on shaker at RT
4	**Dissection**	Of insect brains in PBS
5	**Washing**	3 x in PBS, each wash 10 mins on shaker at RT
6	**Sample storage**	Dehydration in methanol (dilution in PBS): 30%, 50%, 70%, 90%, 100%, 100%, each 10 mins on shaker at RT and rehydration in methanol (dilution in PBS) (90%; 70%, 50%, 30%, 0%, each 10 mins on shaker at RT
7	**Antigen retrieval**	Incubate in sodium citrate buffer (10 mM, pH 8.5) at 80°C 20 mins and wash three times (each 10 mins) in PBS
8	**Washing**	2 x in PBST (0.5%), 1 x in PBST (2%), 1 x in PBST (0.5%), each wash 10 mins on shaker at RT
9	**Blocking**	In 5% NGS-PBST-(0.5%) at 4°C on shaker over night
10	**Primary antibody incubation**	Incubate in primary antibody solution (in 5% NGS-PBST (0.5%)) and 0.02% NaN_3) on shaker 6 days at 4°C and 1 day at RT
11	**Washing**	6 x in PBST (0.5%), each wash 10 mins on shaker at RT
12	**Secondary antibody incubation**	Incubate in secondary antibody solution (in 5% NGS-PBST (0.5%)) on shaker at 4°C over night
13	**Washing**	3 x in PBST (0.5%), 3 x in PBS, each wash 10 mins on shaker at RT
14	**Mounting**	In mounting medium for fluorescence between two microscope cover glasses with spacers (8 stacked paper reinforcement rings), which prevent the cover glass from squeezing the tissue

2.2.4.2. Immunofluorescent staining of larvae brains

In case of staining larval brains in first, third and fifth larval instar the protocol in **table 3** had to be adjusted in several steps. The larval brains are extremely delicate and could not be dissected without fixation. I poked holes with a sharp needle into the larvae and fixed the whole larvae for five hours a first time, dissected and fixed the brains a second time. Adding the second fixation was crucial. In preliminary experiments without the second fixation, a few brains got lost during the staining process (**Tab. 4**). In these cases, probably an insufficient amount of fixative reached the brains through the holes in the larva body. In experiments with bee larvae, no shaker was used except in the fixation step, because they tended to stick together after shaking. I dissected the brains with forceps with thin, but blunt tips, which were slightly curved to the insight. This way I could remove the sticky trachea without damaging the brains. For dissection, I removed the mandibles and opened the head capsule along the neck side. Afterwards I pulled the head capsule from back to front and laid the larva brain open. Then I removed all surrounding tissue and last separated the brain (and in some cases the suboesophageal ganglion attached to the brain) from the thoracic ganglion.

Table 4: Protocol for fluorescent staining of larval bee brains.
Procedure used in experiments with *A. mellifera* (larval instars L1, L3 and L5). The concentration of antibodies is indicated in material (chapter 2.1.2.). For SYN and PDF double staining in experiments on different developmental stages secondary antibodies Alexa Flour 488 goat anti mouse and 635 (555 in preliminary experiments) goat anti rabbit were used.

Step	Method	
1	**Pre-fixation preparation**	poke holes with a sharp needle into the larvae
2	**First fixation**	Incubate larvae in 4% PFA in PBST (0.1 %) on shaker 5h at RT
3	**Washing**	3 x in PBS, each wash 10 mins at RT
4	**Dissection**	Of insect brains in PBS with forceps
5	**Second fixation**	Incubate brains in 4% PFA in PBST (0.1 %) 1h at RT
6	**Washing**	3 x in PBS, each wash 10 mins at RT
7	**Washing**	3 x in PBST (0.5%), each wash 10 mins at RT
8	**Blocking**	In 5% NGS-PBST-(0.5%) at 4°C over night
9	**Primary antibody incubation**	Incubate in primary antibody solution (in 5% NGS-PBST (0.5%)) and 0.02% NaN_3) 3 days at 4°C and 1 day at RT
9	**Washing**	6 x in PBST (0.5%), each wash 10 mins at RT
10	**Secondary antibody incubation**	Incubate in secondary antibody solution (in 5% NGS-PBST (0.5%)) at 4°C over night
11	**Washing**	3 x in PBST (0.5%), 3 x in PBS, each wash 10 mins at RT
12	**Mounting**	In mounting medium for fluorescence on microscope slide

2.2.4.3. Sample storage

In most cases, it was not possible to collect bee brains for one experiment all together and the samples had to be stored in 100% methanol at -20°C until immunofluorescent staining of the brains. This step was performed as indicated in **table 3** after step 5 in the staining protocol. In a series of increasing methanol concentration in PBS medium (30%, 50%, 70%, 90%, 100%, 100%) the brains were dehydrated and proteins were stabilized. Before continuing the protocol brains were rehydrated (90%; 70%, 50%, 30%, 0% methanol in PBS) and rinsed three times in PBS.

2.2.4.4. Vibratome sectioning

For microscope pictures with a higher magnification the whole mount bee brains were sectioned with the vibratome (Leica, VT1000 S) in frontal sections of either 150 µm or 60 µm thick slices. Therefore, the whole mount brains were embedded in 7% agarose (low EEO, agarose standard, AppliChem) and the razor blade (Wilkinson) was positioned in a 10° angle with vibratome speed and frequency of 5 and 6 (on the scale). The sectioning was performed subsequent to step 7 in the staining protocol (**Tab.3**). Further adjustments to the staining protocol of sectioned brains: In washing step 6 no 2% PBST was used and the incubation times of primary antibody was shortened to 3 days at 4°C and 1 day at room temperature. The concentration of anti-*am*PER antibody varied: 1:1000 in 60 µm sections and 1:100 (pre-absorbed) in 150 µm sections. Mounting of the sections was performed with mounting medium for fluorescence on a microscope slide.

2.2.4.5. Antigen retrieval

I added an antigen retrieval step (step 7 in the staining protocol (**Tab.3**) in order to gain a better fluorescent signal, which was not necessary for staining larval brains. The whole mounts were incubated in sodium citrate buffer (10 mM, pH 8.5) at 80°C in a water bath (Memmert, W 200) for 20 mins and washed three times (each 10 mins) in PBS.

2.2.4.6. Double staining in a row (PER and PDF double staining)

Some of the primary antibodies used in this thesis are raised in the same animal, wherefore I could not perform a staining of the recognized antigens at the same time. To analyze the combined localization of their target proteins in the insect brain I performed the whole protocol for staining each protein/peptide one after the other. For this double staining in a row I completed the whole staining until step 13 in the staining protocol (**Tab.3**) for one primary antibody (anti-*am*PER), fixed the brains again (Zamboni's fixative) at 4°C over night and washed them thoroughly (in PBS, 10 times 10 mins each). Then I repeated the staining protocol from step 8 on (**Tab.3**) for the second primary antibody

(anti-βPDH). Anti-*am*PER antibody was labeled with Alexa Fluor 488 goat anti rabbit and anti-βPDH antibody with Alexa Fluor 635 goat anti rabbit.

Basically the same procedure was applied to *Drosophila* brains, but with shortening of the incubation times due to the smaller size of these brains (**Tab. 5**).

Table 5: Protocol for fluorescent double staining in a row in whole mount brains of *D. melanogaster.*
Procedure used in experiments to validate the new anti-*am*PER antibody. It follows basically the double staining in a row protocol for bee brains, but I used pre-absorbed anti-*am*PER antibody (1:100) and shortened the primary antibody incubation times.

Step	Method	
1	**Pre-fixation preparation**	Immobilize flies on ice
2	**Fixation**	Incubate in 4% PFA in PBST (0.1 %) on shaker 3-4h at RT
3	**Washing**	3 x in PBS, each wash 10 mins on shaker at RT
4	**Dissection**	Of insect brains in PBS with forceps
5	**Washing**	3 x in PBS, each wash 10 mins at RT
6	**Sample storage**	Dehydration in methanol (dilution in PBS): 30%, 50%, 70%, 90%, 100%, 100%, each 10 mins on shaker at RT and rehydration in methanol (dilution in PBS) (90%; 70%, 50%, 30%, 0%, each 10 mins on shaker at RT
7	**Antigen retrieval**	Incubate in sodium citrate buffer (10 mM, pH 8.5) at 80°C 20 mins and wash three times (each 10 mins) in PBS
8	**Washing**	3 x in PBST (0.5%) each wash 10 mins at RT
9	**Blocking**	In 5% NGS-PBST (0.5%) over night at 4°C
10	**Primary antibody incubation**	Incubate in primary antibody solution of anti-*am*PER (pre-absorbed 1:100) in 5% NGS-PBST (0.5%) and 0.02% NaN_3) 2 days at 4°C and 2 day at RT
11	**Washing**	6 x in PBST (0.5%), each wash 10 mins at RT
12	**Secondary antibody incubation**	Incubate in secondary antibody solution (Alexa Fluor 488 goat anti rabbit in 5% NGS-PBST (0.5%)) at 4°C over night
13	**Washing**	3 x in PBST (0.5%), 3 x in PBS, each wash 10 mins at RT
14	**Fixation**	With Zamboni's fixative over night at 4°C
15	**Washing**	With PBST 10 times each 10 mins at RT
16	**Washing/blocking**	repeat step 8 and 9
16	**Primary antibody incubation**	Incubate in primary antibody solution of anti-βHRP (1:3000) in 5% NGS-PBST (0.5%) and 0.02% NaN_3) 4 days at 4°C and 1 day at RT

	Washing/secondary antibody incubation	repeat step 11-13, but use as secondary antibody Alexa Fluor 635 goat anti rabbit
17	**Mounting**	In mounting medium for fluorescence

2.2.4.7. Pre-absorption of antibodies

In order to reduce unspecific binding, the antibodies (anti-*am*PER) were pre-absorbed on *Drosophila* mutant *per*^{01} embryos following a method adapted from Rothwell and Sullivan (2000). The pre-absorbed antibody was used in experiments with *D. melanogaster* (1:100), in vibratome sections of honey bee brains (1:100) and for labeling PER in whole mount brains of *O. bicornis* (1:1000) and *O. cornuta* (1:500). (Note that anti-*am*PER in experiments with whole mount brains of *A. mellifera* has not been pre-absorbed and used in a concentration of 1:1000). For the pre-absorption procedure fly eggs were collected on apple juice agar plates (with moist yeast on top) over night, washed with H_2O dest. and dechorionized with bleach. I washed the embryos in H_2O dest., PBST(0.5%), H_2O dest. (each 5 mins on shaker) and fixed them for 5 mins in a mixture of 37% formaldehyde and heptane (1:1). After removing the lower phase (formaldehyde) methanol (100%) was added, the mixture was shaken thoroughly for 15 seconds and then incubated at room temperature until phases were separated. Only the embryos in the bottom part of the lower phase (methanol) were devitellinized and everything else was removed. I rehydrated these embryos in two steps (50% methanol/PBST(0.5%), PBST(0.5%), each 5 mins on the shaker), washed two times in PBST(0.5%) and incubated them in the antibody solution (1:100 in 5% NGS-PBST(0.5%) and 0.02% NaN_3) for at least 1 hour on the shaker at RT.

2.2.4.8. DAPI (4', 6-diamidino-2-phenylindole) staining

Nuclei were counterstained with DAPI fluorescent stain (SIGMA-Aldrich) which intercalates into the DNA helix and is excited by UV-light (400 nm). For DAPI staining step 11 in the staining protocol (T**ab.3**) was altered: The fifth washing was performed with 1 mg/ml DAPI in PBS and extended to 20 mins. After three more washes in PBS the brains were mounted.

2.2.4.9. HRP (Horseradish peroxidase) staining

I performed a direct staining method specific for neurons (Loesel et al., 2006) with anti-HRP antibody already conjugated to a fluorophore. Anti-HRP antibodies recognize in insects a carbohydrate epitope of protein components called Nervana (Nerve antigen) (Sun and Salvaterra, 1995). In the central nervous system anti-HRP antibody labels these components in neuronal membranes. Therefore the somata of neurons and the neuropiles are clearly illuminated in immunofluorescent staining (Jan and Jan, 1982; Loesel et al., 2006). After staining was completed with the last washing step brains were blocked over night at 4°C and then incubated in the HRP antibody solution (1:300 in 5% NGS-PBST (0.5%)) 48h at room temperature on a shaker. Afterwards the brains were washed again (3 x in PBST (0.5%) and 3 x in PBS) and mounted in mounting medium for fluorescence.

2.2.4.10. Data analysis

Immunohistochemistry data was analyzed with a Leica TCS SPE confocal microscope (Leica microsystems, Wetzlar, Germany) equipped with 10x/0.30 CS ACS APO and 20x/0.60 IMM CORR ACS APO objectives. Brains were scanned sequentially with different lasers (405nm, 488nm, 532nm, 635nm) in stacks whereby bee brains had to be scanned in several stacks in xy-direction, because of their size and work distance limitations of the objective. Images were stitched together afterwards with the stitching tool of the Leica software (Leica Application Suite Advanced Fluorescence 2.7.3.9723, Leica Microsystems, Mannheim, Germany). Moreover, the laser could not fully pass the brain tissue of whole mount bee brain preparations in z-direction, wherefore I scanned anterior and posterior half of the brains separately. Samples of one experiment were processed with the same settings and obtained confocal pictures (resolution 1024 x 1024) were further processed in Fiji ImageJ (Version 1.49, © Wayne Rasband, National Institutes of Health, USA). Fluorescent signals were investigated regarding neuroanatomical location, morphology and number of stained cells. In not yet manipulated confocal picture stacks, quantification of cell number was done by counting stained cells in both hemispheres and then averaging for the brain. In case of the cluster analysis for PDF staining, I quantified the two hemispheres separately. Only brightness and contrast were adjusted in the figures displayed in the results section.

2.2.4.11. 3-D Reconstruction of the PDF arborizations in the bee brain

I used the software Amira (Version 6.1.1; FEI Visualization Sciences Group, Zuse Institute Berlin) for the 3-D reconstruction of the PDF network in bee brains. Before starting the reconstruction the voxel size of the z-direction had to be corrected (factor 0.6 – 0.7), because the scan was not performed in the objective specific optimal step size (2.39 µm for 10X objective and 0.89 µm for 20 X objective). Anterior and posterior halves of the brains were then aligned in the Amira software with the “transform editor” by matching overlaying structures. I reconstructed the neuropils using the “segmentation editor” with manually assigning voxels to neuropils. After subsequent resampling, a smoothed surface 3-D model was generated and displayed in the “surface view”. The PDF fibers were reconstructed using the “filament tracer” tool in which I marked manually the beginning, branching and end points of stained fibers and the program automatically generated the connections based on differences in voxel brightness. In the “spatial graph view” the 3-D fiber reconstruction was aligned with the reconstructed neuropils. The PDF fibers showed many varicosities with faint PDF staining in between. In dense fiber tracts, this occasionally resulted in false cross connections between single fibers. I avoided these calculation errors by selecting a high number of tracing points along the fibers. This way I visualized the main characteristics of the PDF network, with the compromise of maybe missing a few details. For example, the varicosities are not preserved in the neural network reconstructions. I generated a 360° rotation animation (function “camera orbit (rotate)”) of the final reconstruction and took a picture

sequence of it with the “movie maker”, which can be merged and converted to a movie format in Fiji ImageJ (Version 1.49, © Wayne Rasband, National Institutes of Health, USA).

2.2.5. Statistics

Statistical analysis was conducted in Microsoft Excel (2013 Microsoft Office) and R (R version 3.2.2). Values were defined significantly different (*) at $p \leq 0.05$ and highly significantly different (**) at $p \leq 0.01$ and were indicated by asterisks in all graphs. Performed tests are stated in the corresponding results section.

2.2.5. Nomenclature

I followed the naming conventions for neuropils in insect brains as suggested by Ito and co-authors (Ito et al., 2014). Names for the clock neurons in the bee are adapted from the nomenclature in *Drosophila melanogaster*. I will refer in this thesis to the neurons that are stained with the PER antibody and/or the PDH (PDF) antibody as “PER-positive” and “PDF-positive” neurons or simply “PER and PDF neurons”, respectively. Similarly, I refer to fibers arising from the PDF neurons, as “PDF fibers”/”PDF arborizations”/”PDF network”.

3. Results

3.1. A new device for monitoring individual activity rhythms of honey bees reveals critical effects of the social environment on behavior (First author paper)

In a newly developed set up for measuring activity of honey bees in social context (described in 2.2.2.1.) I could show that indirect social contact of individually monitored bees to a mini colony has effects on their survival and locomotor rhythms. To ensure a comparable function of the mini colony in the laboratory and a natural bee colony we kept the mini colony for three weeks in the incubator and checked its status afterwards. I detected that brood was raised normally, no dead brood was lying in the box, brood cells were opened and young bees were present in the mini colony. Furthermore, only old bees, that had been foragers at the beginning of the experiment, had died during this time, which may have coincided with their natural time of death.

I was possible to record the mini colony's rhythm by measuring the temperature inside the mini hive, whereby the highest amplitude in temperature rhythm (28 - 33.4°C) was registered close to the food storing cells (sensor "food", **Fig. 8 A**) and lower amplitudes at the center of the brood and in a monitor tube directly besides the mini hive (sensor "brood" and "attached monitor", **Fig. 8 A**). Overall temperature was higher at the brood (31.1 – 34.8°C) and average temperature was lower in the attached monitor. Constant temperatures of 28°C, which matched the incubator settings, were registered in the tubes of the monitor without contact and inside the incubator (**Fig. 8 A**). In constant conditions we could see the colony rhythm free run in parallel with the activity of forager-aged bees (period τ of 23.9 (±0.4) h, no significant differences between τ of the colony and the individual bees: $p > 0.5$), but the temperature peaked slightly earlier than the bees' activity (**Fig. 8 B**).

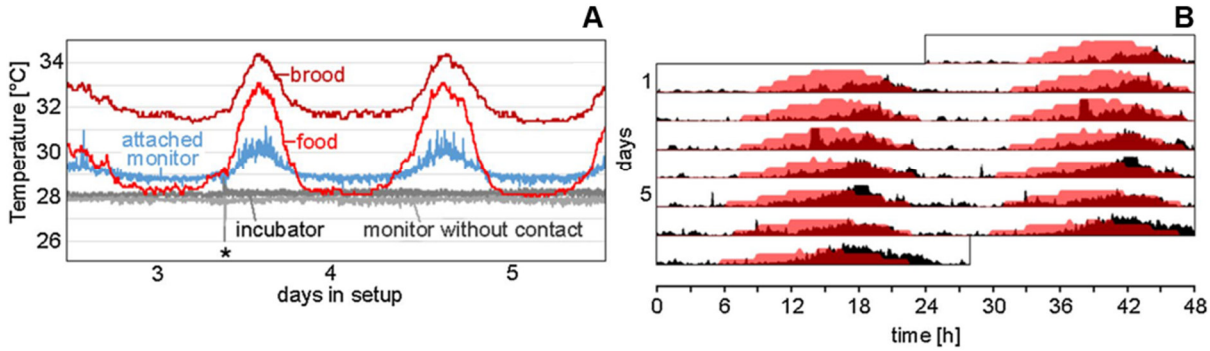

Figure 8: Monitoring of the colony rhythm via measuring temperature.
A: Temperature data of sensors located at different places in the set up. The asterisk indicates that incubator was opened to refill food provisions for the mini colony. **B**: Actogram (double plot) of circadian average activity of forager-aged bees (black, n = 7) in social contact with the mini colony plotted together with the colony rhythm measured by temperature oscillations (red, data from sensor "food"). Incubator settings: 28°C, 60% RH, DD. (adapted from (Beer et al., 2016))

When I compared survival of bees in monitors with social contact to bees in monitors without social contact (0.5 m away from the mini hive), I detected that isolated newly emerged bees had a significant

higher number of death incidents than newly emerged bees with social contact (survival of "nurse-aged bees" on the first day in the set-up, Fisher's exact test, p=0.001, Fig. 9 A). This difference was only observed for newly emerged bees i.e. for nurse-aged bees on the first day in the set-up while newly emerged bees with social contact showed a survival rate similar to that of older forager-aged bees (Fig 9 B). These two survival experiments were performed under DD, but I also got similar results for the survival of newly emerged bees with or without social contact under LD cycles (Fisher's exact test, $p<0.05$, Fig. 9 C) with slightly different incubator settings.

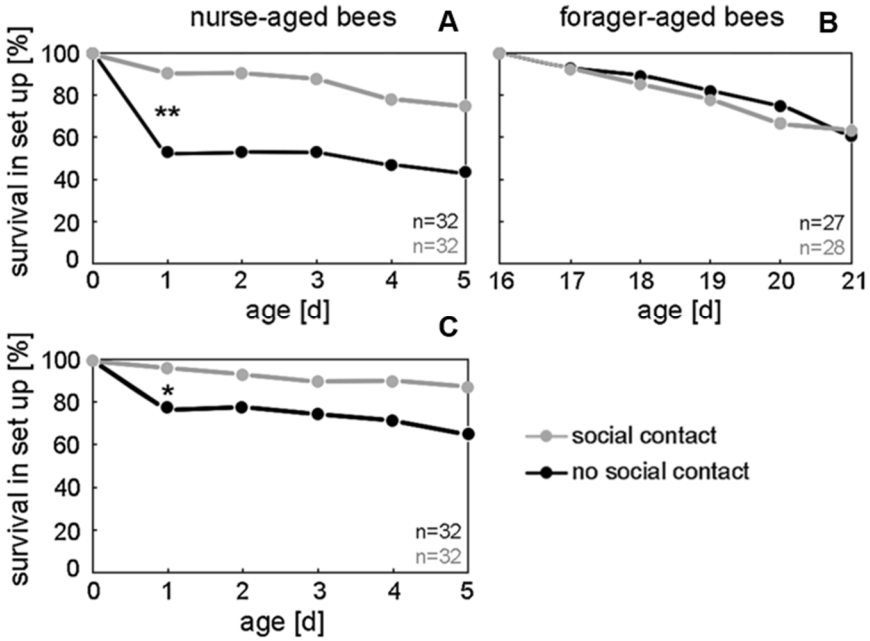

Figure 9: Survival of individually monitored honey bees.
A: Newly emerged bees (nurse-aged bees) without social contact to the mini colony show significant difference in survival on the first day compared to nurse-aged bees that are in social contact. These display a similar survival rate to forager-aged bees (**B**) in the set-up. Incubator settings: 30°C, 60% RH, DD. In another experiment with slightly different environmental settings (14:10 LD cycle and 28 °C) I observed the same difference in survival between newly emerged bees with or without social contact on day 1 in the set-up (**C**) (adapted from (Beer et al., 2016))

In an experiment with conflicting Zeitgebers of light and social contact, I determined if one could observe social synchronization in my new set-up. Therefore I exposed the bees in the monitors to a LD cycle that was phase shifted by approximately 10 h to the natural LD cycle (sunrise: 05:37 o'clock; sunset: 20:56 o'clock) that bees (mini colony and forager-aged bees) were entrained to. I observed that forager-aged bees without social contact shifted their main activity significantly faster ($p < 0.001$, Wilcoxon rank sum test) into the light phase than forager-aged bees with social contact, which need almost double the time (6 (±0.2) days instead of 3 (±0.3)). Because of some residual light entering the mini hive at the openings to the monitor tubes, the colony received also a light signal, but it functioned as a weaker Zeitgeber than the strong illumination of the monitor tubes. Therefore, the colony rhythm shifted slowly and forager-aged bees with social contact shifted their activity with the same pace (**Fig. 10**). Nurse-aged bees with social contact needed on average only 2.5 (±0.4) days to show activity

rhythms in their behavior whereas young bees without social contact need 3.9 (±0.5) days which is significantly longer (($T_{(17)}$ = 2.31, p = 0.03)) (**Fig. 10**). Even after having developed a rhythm, the power of rhythmicity of nurse-aged bees is significantly lower than of forager-aged bees and, overall, fewer bees are rhythmic compared to the forager-aged bee observations (**Tab. 6**). There is no difference in power of rhythmicity between the groups in social contact or without social contact. Inspecting the daily activity profile on day 7 (**Fig. 11**) in the set-up revealed that the test subjects that were socially isolated displayed acrophases that were significantly different from the colony's temperature peak, but the acrophases of forager-bees with social contact were not. In case of the nurse-aged bees in social contact, I determined two separate activity peaks in 7 of 16 individuals.

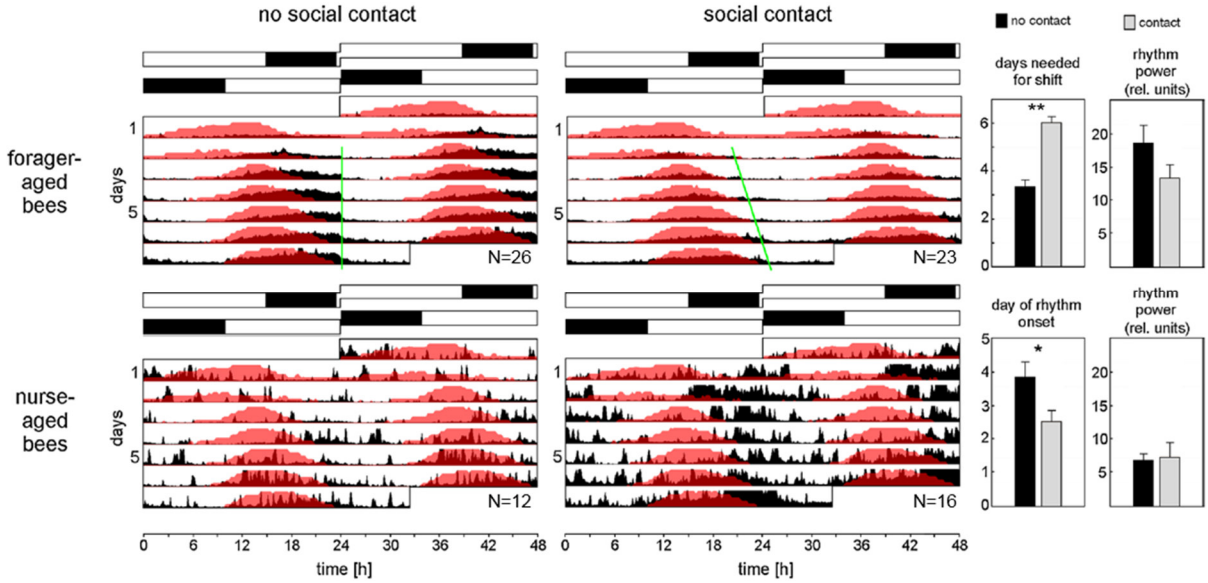

Figure 10: Average actograms (double plot) of individually monitored honey bees and temperature rhythm of the mini colony.
Bees were of different age groups (forager- and nurse-age) and have either social contact to a mini colony or not. Light bars on top of the actograms indicate the natural entrainment LD cycle and the shifted LD cycle during the experiment. Forager-aged bees without social contact shift their activity (black) significantly faster (green line and graph) than forager-aged bees with social contact. The latter shift their activity at the same pace as the temperature rhythm of the colony (red), meaning their shift is in parallel to the colony's rhythm shift. Nurse-aged bees with social contact show an onset of rhythmicity on average 2 days earlier than the young bees without social contact. (adapted from (Beer et al., 2016))

A new device for monitoring individual activity rhythms of honey bees reveals critical effects of the social environment on behavior (First author paper)

Table 6: Percentage of rhythmic bees and their rhythm power.
Rhythmicity and power of rhythmicity for bees (N=number of evaluated individuals) of different age ("Forager-aged bees" and "Nurse-aged" bees) in the two treatments "with social contact" to a mini colony and "without social contact".

	No social contact			**Social contact**		
	N	Rhythmicity (%)	Power of rhythmicity (rel. units)	N	Rhythmicity (%)	Power of rhythmicity (rel. units)
Forager-aged bees	26	84.62	18.63 ± 2.69	23	95.65	13.51 ± 1.88
Nurse-aged bees	12	58.33	6.93 ± 1.02	16	75.00	7.29 ± 1.16

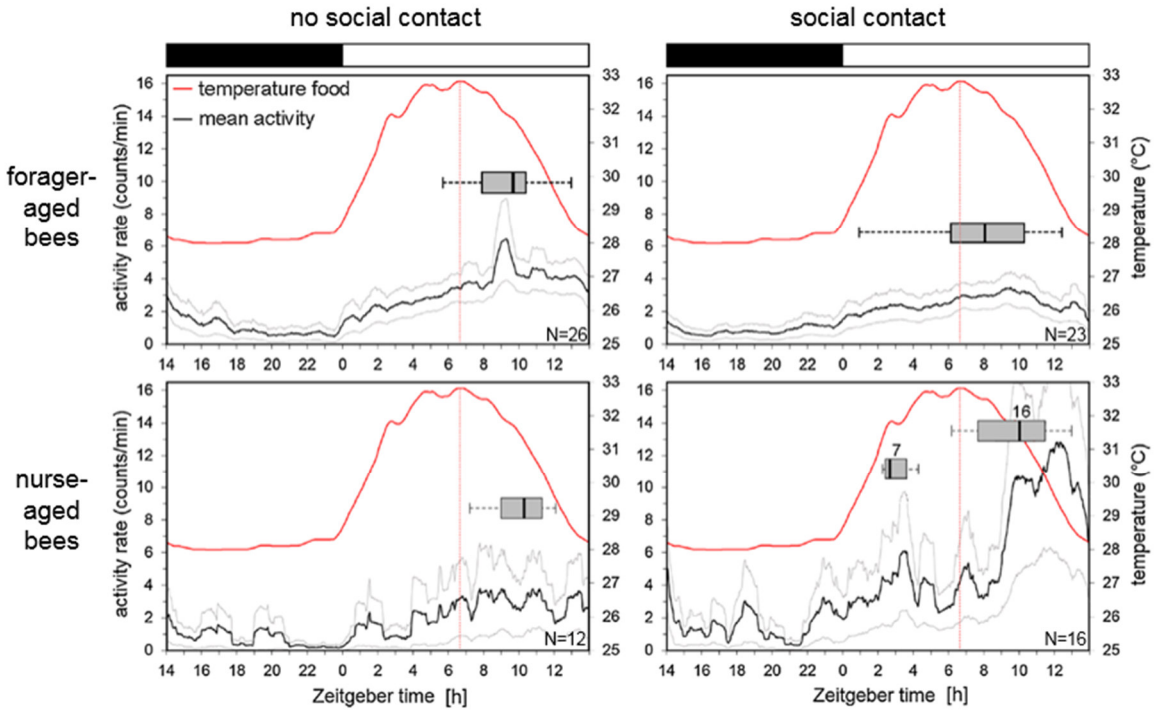

Figure 11: Mean activity profile of monitored bees and temperature profile on day 7 in the set-up.
Average activity of bees (black) (grey = standard error) plotted together with the temperature profile of the mini colony (red). Acrophases of the activity is displayed as boxplots (forager-aged bees and nurse-aged bees without contact). 7 of 16 nurse-aged bees with social contact showed 2 separate activity peaks. Light regime is displayed as LD bar above the graphs. (adapted from (Beer et al., 2016))

3.2. Activity rhythms in solitary bees: Emergence and locomotor activity

Emergence rhythms in solitary bees

The best studied rhythms in solitary bees are the annual rhythms of emergence in spring. In a collaboration project with Mariela Schenk and Andrea Holzschuh (University of Würzburg) we wanted to determine if emergence rhythms of *O. bicornis*, a bee wintering as imago, are governed by the circadian clock and which environmental triggers might entrain the circadian clock. I monitored circadian emergence of *O. bicornis* under constant dark conditions and Mariela monitored emergence under LD cycles with constant temperature and under cycling temperature with constant darkness. From preliminary studies and from experiments with pre-pupae overwintering species I knew that temperature cycles can be strong synchronizing factors. Therefore, I conducted entrainment in temperature cycles (as described in chapter 2.2.2.2.) and kept temperature at constant high (19.2°C ± 0.1 °C) during emergence time. 23 bees of the 209 inserted cocoons did not emerge or were infested by parasites, 3 bees emerged before start of constant conditions and 2 bees emerged after day 13 which we set as the end of the experiment. These 28 bees were excluded from analysis, which was conducted on the remaining 181 (92 males and 89 females) animals. By inspecting the raw data binned in 2h time resolution I could already detect a circadian rhythm by eye which was confirmed by the autocorrelation analysis (autocorrelation function (acf), 30 min data bin, conducted in R) over 13 days (**Fig. 12 .A/B**). Significant cycling in emergence was indicated by the bouts surpassing the confidence interval (blue line at 0.05) in the acf. The whole population showed significant circadian cycling during the first five days and 2-3 days on which emergence did not surpass the confidence interval, but still fit to the rhythm pattern. Males (**Fig. 12 B1**) had a similar rhythm with 5 significant circadian peaks, while females showed only 3 (**Fig. 12 B2**).

Males emerged approximately 4 days earlier (mean emergence day of males: 4.0 ± 0.3, and of females: 8.2 ± 0.3; $p<0.01$, Wilcoxon rank sum test) and in the emergence actogram (**Fig. 12 C**) the main activity of the temperature raise signal was easy detectable during the first 7 days after releasing the bees into constant conditions. From day 8 onward, no rhythmicity is detectable by eye anymore. In the periodogram analysis, which was conducted for the whole 13 days (Chi-Square test, smoothing factor 10) with the data binned in 1 min intervals, the whole population had an emergence FRP of exactly 24h, for the male subpopulation it was the same (FRP: 1440 min) and females were slightly faster (FRP: 1405 min). The power of rhythmicity was stronger for the emergence in the male subpopulation (1503 relative units) than for females (773 relative units) (whole population: 1473 relative units). I used for the evaluation different data bin sizes because visualization of rhythms is better in 2h data bins and most exact resolution for the analysis of FRP is achieved with 1 min data bins. For the autocorrelation analysis, I chose a 30 min data bin, because this was the highest resolution achieved in the parallel experiment conducted by Mariela. This way we could compare our results.

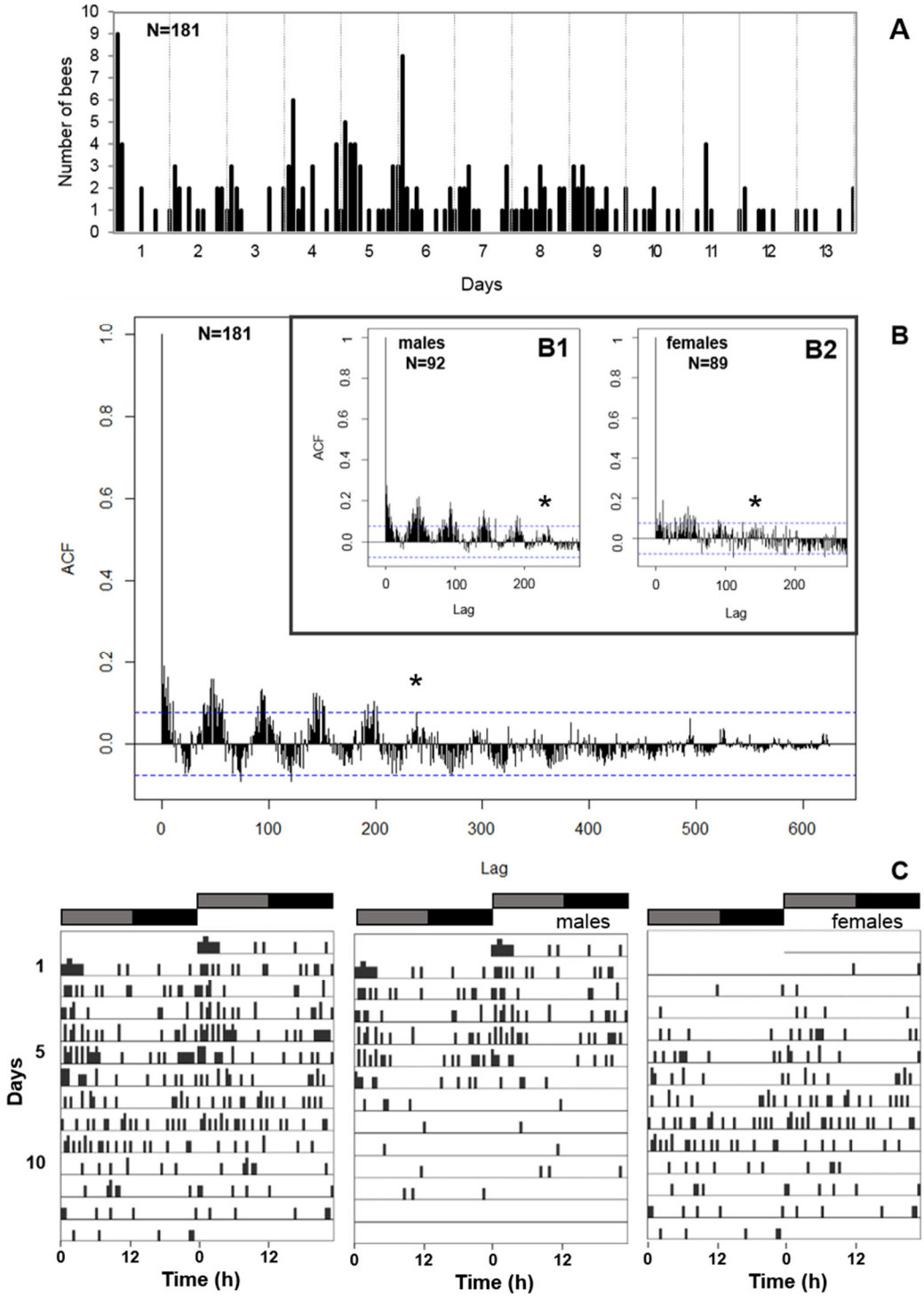

Figure 12: Circadian emergence rhythm of *O. bicornis* population after entrainment in temperature cycles (12:12).
A: Histogram of emergence incidence in constant temperature and darkness with data binned in 2h time intervals. X axis depicts time passed after release into constant conditions and grey vertical line marks 24 h intervals in this time line. **B:** Autocorrelation function (data binned in 30 min = unit of time lag) of 13 days shown in A, for whole population and males (**B1**) and females (**B2**) respectively. Asterisks mark the last peak in the circadian rhythm pattern surpassing the confidence interval (blue dotted horizontal line), which indicates circadian cycling for several days. X axis shows the time lag and indirectly passed time since last temperature signal (at lag 0). **C:** Emergence rhythm in DD plotted as emergence actogram (double plotted, time of the day in hours (hour 0 at day 1 = time of last temperature signal)) for days 1-13. Environmental bars above: light phase (grey) and dark phase (black) during previous entrainment.

In our parallel experiment with Zeitgebers temperature and light, conducted by Mariela Schenk, we found out that the entrainment of circadian emergence rhythms of *O. bicornis* is highly sensitive to temperature and insensitive to LD signals. Entrainment was achieved by a single temperature pulse as low as 1 °C while light had no visible effect on emergence rhythmicity (see supplementary figures from joint study, **S1-2**).

Locomotor activity rhythms in solitary bees

In case of locomotor activity rhythms of the adult solitary bees I could show that these are entrainable by LD cycles (**Fig. 13**). In a preliminary experiment I entrained newly emerged mason bees (*O. bicornis*) (N=5) in one group to a LD (12:12) cycle that was phase shifted by 10 hours to the natural light dark cycle and transferred them after 3 days into the monitors in DD to measure their circadian rhythmicity. The bees show their main activity on the first day in constant conditions (DD, 20 ± 0.5 °C) at the same time of the day, the light phase has been before. The light entrainment of 3 days appeared to be very effective in males (**Fig. 13 A, left**) and less efficient in females (**Fig. 13 A, right**) which have a less clear differentiation between activity and rest phase in their activity profile. In addition, free-running period (FRP) in activity of some female bees was less stable in DD and switched from a long period (more than 24h) to a short period (less than 24h). In another experiment, I exposed solitary bees (N=12) to two differently phased light regimes with a period of constant conditions (DD) in between and afterwards. Again, in all cases the bees followed the light entrainment and showed their main activity phase on the first day in DD during the time of the day the light phase had been in the previous LD cycle. (**Fig. 13 B**). The bees showed either phase advances or delays in their locomotor activity rhythms after exposure to LD depending on which subjective phase they were in before the illumination regime changed. In the first example (**Fig. 13 B, left**) the second LD occurred in the late subjective night of the bee and the animal advanced its activity. In comparison, the bee in the second example (**Fig. 13 B, right**) phase delayed its activity, because illumination changed during its early subjective night. Also in this experiment, I observed highly various FRPs ranging from 21.7 h to 27.0 h, whereby predominantly females showed FRPs longer than 24 h. Since we had observed extremely high temperature sensitivity of the mason bees in emergence experiments (see supplementary figures from joint study, **S1-2**), I controlled for temperature variations during the experiment with an additional data logger with higher data resolution (± 0.1 °C instead of ± 0.5 °C). The temperature in the incubator was kept at exactly 19.7 °C with a standard deviation of 0.1 °C. This means that a temperature is unlikely to be the cause of the observed change in period.

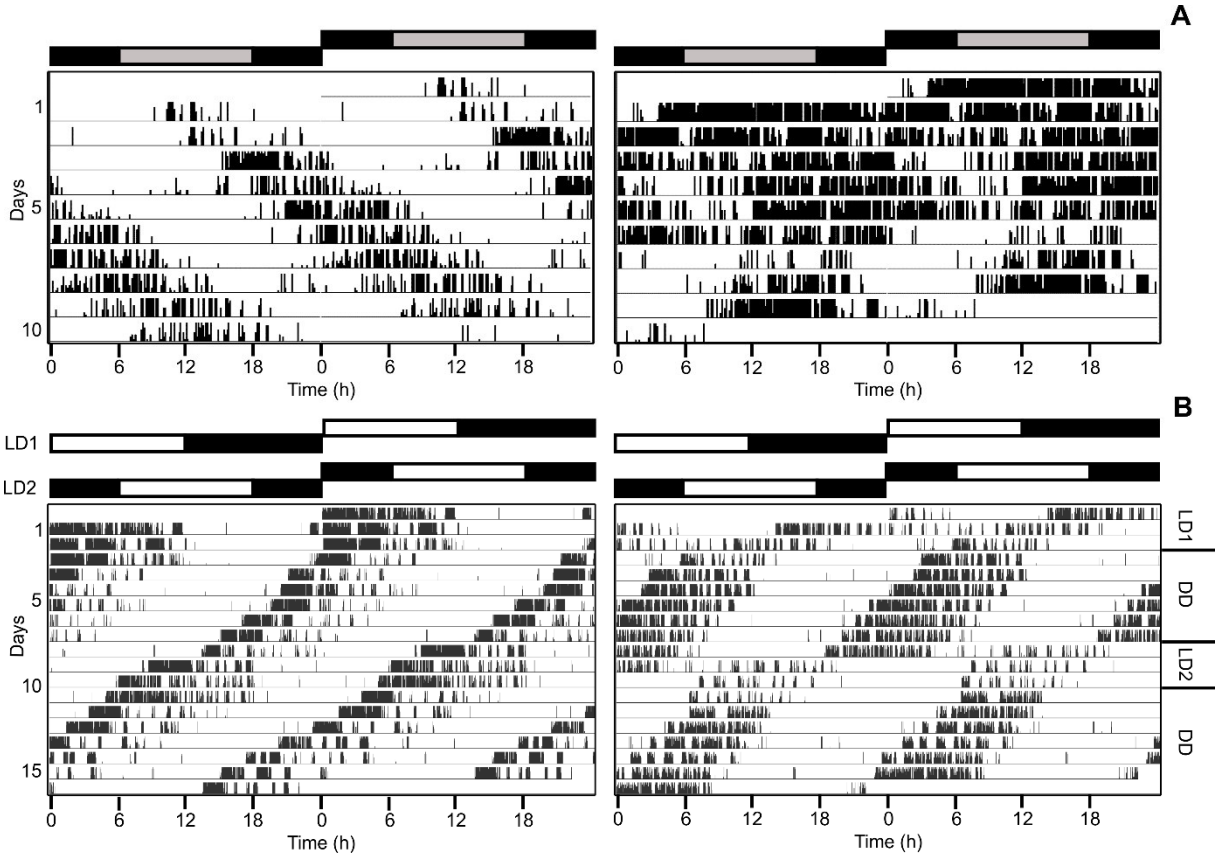

Figure 13: Light entrainment of adult *O. bicornis* bees.
Representative double plotted actograms of locomotor activity during constant conditions after subjecting the bees to one (**A**) or two LD (LD1 and LD2) regimes of 12 hours light and 12 hours darkness (**B**). **A:** male (**left**) and female (**right**) mason bee in DD after light entrainment. Environmental bars on top depict the entrainment light cycle (light grey: light phase; black: dark phase) that had been applied before day 1 in the actogram. **B:** bees follow the light entrainment in LD2 by either phase advancing their activity (**left**) or delaying it (**right**). Environmental bars on top depict the entrainment light cycle in LD1 and LD2 (white: light phase; black: dark phase). The LD cycles are followed by several days in constant conditions (DD) which is marked in a timetable to the right of the actograms.

3.3. Neuroanatomical characterization of the location of PER and PDF in social and solitary bees

Spatial expression pattern of *period* in *A. mellifera* (co-authored manuscript, see attachment)

A newly produced antibody raised against the entire *amperiod* gene was used in neuroanatomical studies in *A. mellifera* brains. One of the first steps in the study was to verify the antibody's specificity. We used three different approaches in cooperation with Eva Winnebeck (University of Auckland, New Zealand) and Guy Bloch lab (Hebrew University, Jerusalem, Israel): Specificity tests via IP, Western Blot time course analysis (see Appendix 7.5 and 7.6) and I tested for a possible recognition of PER in *D. melanogaster* brains (wildtype CS and null mutant per^{01}). In the wildtype brains several PER-positive neurons were stained with the anti-*am*PER antibody that correspond to the PER-positive clock clusters described before with an antibody raised against *Drosophila* PER (Helfrich-Förster et al., 2007a) (**Fig. 14, first row**). This was true in all 16 wild-type whole-mount brains; but I never saw these cells in any of the 10 simultaneously stained brains of per^{01} mutants (**Fig. 14, second row**). This indicates that the anti-*am*PER antibody recognizes specifically PER. In addition to the specific staining, I found small dots of putatively unspecific staining throughout the brain. The unspecific signals are easily distinguishable from the specific nuclear staining in wild-type brains and seem to be more apparent in the per^{01} brains.

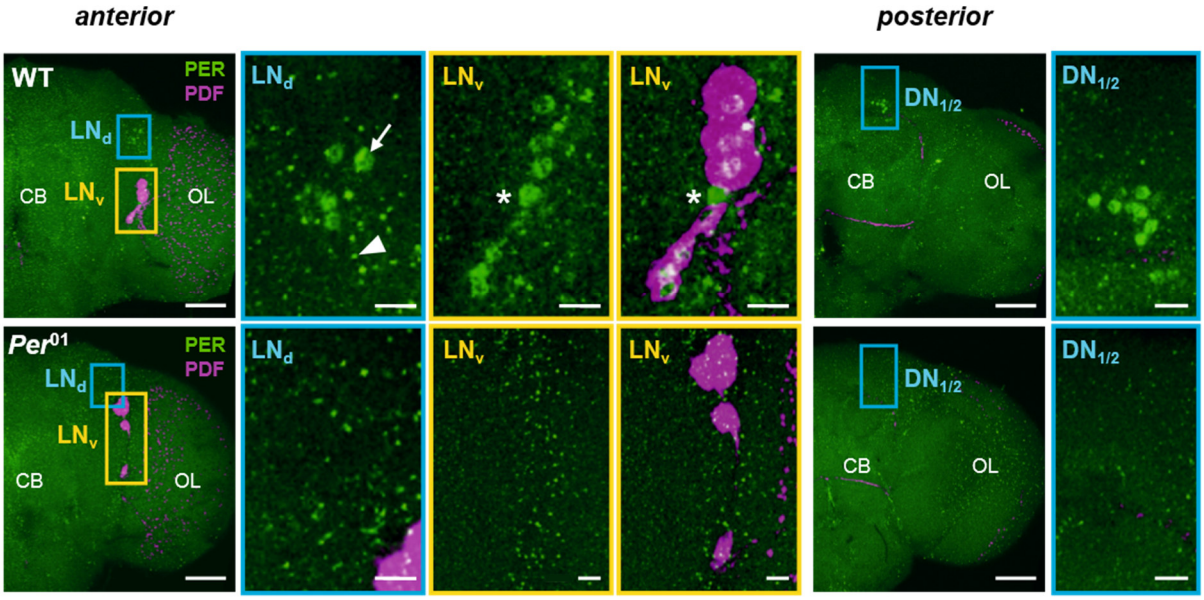

Figure 14: Pictures of double staining of PDF (magenta) and amPER (green) in whole mounts of *Drosophila melanogaster*.
First row: Wild-type strain Canton S. In the whole hemisphere, pictures of the anterior and posterior part of the wild-type brain are the different PER-positive neuron clusters (nuclear staining). These are the LN_d, LN_v and $DN_{1/2}$ marked by a colored frame and displayed in separate detail pictures. In the PER and PDF double staining I could distinguish between the l-LN_v (bigger cell bodies) and stronger cytoplasmic PDF staining and the s-LN_v neurons (smaller cell bodies and weaker staining intensity). The 5th s-LN_v (marked by asterisk) is not PDF positive. Unspecific staining with the anti-*am*PER antibody (arrowhead) appears brighter but is smaller than the specific nuclear staining (arrow). **Second row:** Staining pictures of analogue brain areas in the functional period null mutant per^{01}. Scale bars in hemisphere pictures are 50 µm and in detail pictures 10 µm. WT:wild-type, CB: central brain, OL: optic lobe. Pictures are taken with a 25x objective (0.6 numerical aperture); distance of z-stacks: 1µm (6 overlaid stack in the anterior brain and 22 overlaid stacks in the posterior brain). (Adapted from Fuchikawa et al., 2017)

In the following experiment, we detected several PER-positive cells throughout the honey bee brain and I categorized them into neurons and putative glia with HRP staining (**Fig. 15**). I double stained 17 whole mount brains of forager bees of unknown age with anti-PER and anti-PDH antibody. Half of the bees were dissected immediately after collection (ZT 11), and the other half in the following night (ZT 22) (approximate ambient LD 11:13). In addition, three brains, sampled at ZT 22, were sectioned with the vibratome (60µm (bee entrained to ambient LD: 11:13) and 150µm (bee entrained to ambient LD 16.5:7.5)). I did not see PER staining in brains sampled at ZT 11, as was expected because of the cycling expression of clock genes. PER levels are expected to be high at ZT 22, which I tested in preliminary experiments, and PER levels in *Drosophila* are known to be high at that time point, too (Zerr et al., 1990). Brains sampled at ZT 22 were further triple and quadruple stained with PER, PDF, DAPI and HRP. In the central nervous system of insects, anti-HRP antibody marks a membrane bound protein residue in neurons, while cells of non-neuronal nature are unlabeled. This way I could identify 4 different *am*PER-positive cell clusters as neurons. We categorized them into: “dorsal neurons” (DN), “dorso-lateral neurons” (DLN), “lateral neurons 1 and 2” (LN_1 and LN_2) (**Fig. 15 A-D**).

The DN cluster consist of approx. 15 neurons located between the anterior superior neuropils of the protocerebrum and the medial and lateral calyces (MCA and LCA) of the mushroom bodies (MB). In few cases, I saw 1-2 PER cells with cytoplasmic staining (**Fig. 15 C**), but most PER staining was restricted to the nucleus. The other PER cell clusters are situated between the ventrolateral neuropils (VLNP) of the protocerebrum and the optic lobe. The LN cell cluster are located in a more anterior part of the brain and lie just adjacent to the medulla, while the cells in the DLN are located rather dorsally and more widespread in such a way that the 60-75 neurons can be found in anterior to posterior parts of the brain (see also chapter “Expression pattern of PDF as part of the circadian clock in honey bees”). The LN_1 and LN_2 are often not spatially separated, but we named them differently because of their slightly different location in the brain, cell size and differential co-labeling with PDF. The 14-16 LN_2 neurons lie ventrally of the LN_1. The latter build with about 105-120 neurons the most prominent PER cell cluster, and they seem to be smaller than the LN_2 (**Fig. 16**). Furthermore all LN_2 neurons co-express *per* and *pdf*.

There are several PER cells, which have nuclei of a longish shape and HRP antibody does not bind. This putative glia cells are widespread in the inter-neuropil brain areas, for example pars intercerebralis (PI) and pars lateralis (PL), antennal lobes (AL) and optic lobes (**Fig 17**). Sometimes these glia cells are situated very close to the PER neurons (**Fig. 16 C**). I can therefore not exclude that there might be some PER-positive glia cells among the PER neurons, when they are clustered closely together.

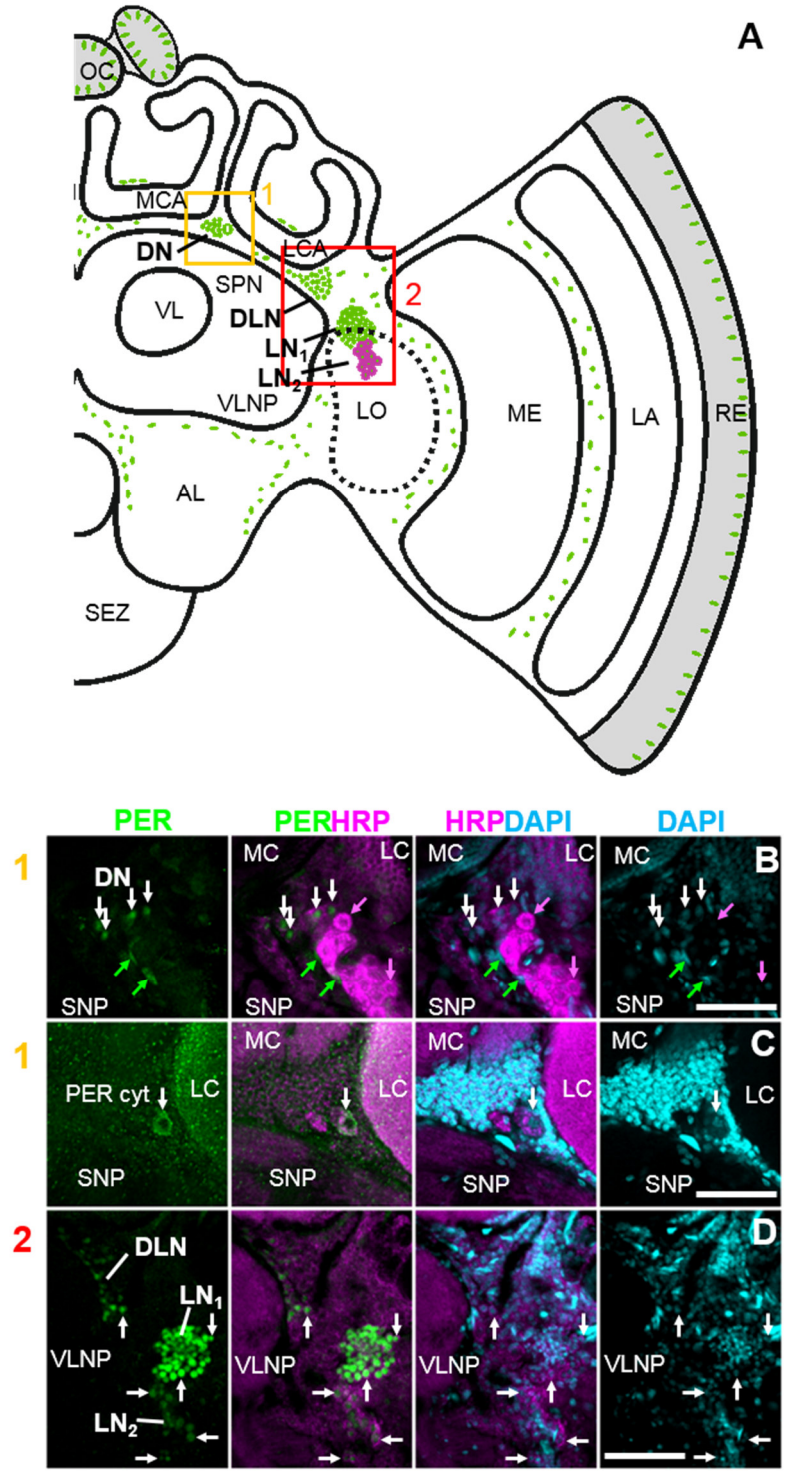

Figure 15: PER-positive staining in neurons in the honey bee brain.
A: Schematic overview of PER cells (green) in one hemisphere. Between the lateral protocerebrum and the medulla (ME) and lobula (LO), which lies posterior and is therefore marked with dotted lines, there are 3 clusters of PER-positive cells (DLN, LN_1, LN_2) and another cluster (DN) in the dorsal brain between medial and lateral calyx (MCA and LCA) and the superior neuropils (SNP). PER neurons double stained with the neuronal marker horseradish peroxidase (HRP): **B-D**. PER neurons consist of the clusters DN (**B** area indicated as 1 in **A**) and 1-2 cells with cytoplasmic PER-positive signal (not double labeled by nucleotide staining with DAPI) (**C** area indicated as 1 in **A**) slightly posterior of this cluster in the dorsal brain. LN_1 and LN_2 neurons are located lateral of the ventrolateral neuropils (VLNP) and DLN cluster in the dorsal lateral brain (**D** area indicated as 2 in **A**). OC: ocelli, RE: retina, VL: vertical lobe, AL: antennal lobe, SEZ: subesophageal zone. All photos in **B-D** were taken with a 10 x objective (numerical aperture: 0.3); distance of z-stacks 2.5; overlay of 3 stacks. Scale bars: 30 µm. (Adapted from Fuchikawa et al., 2017)

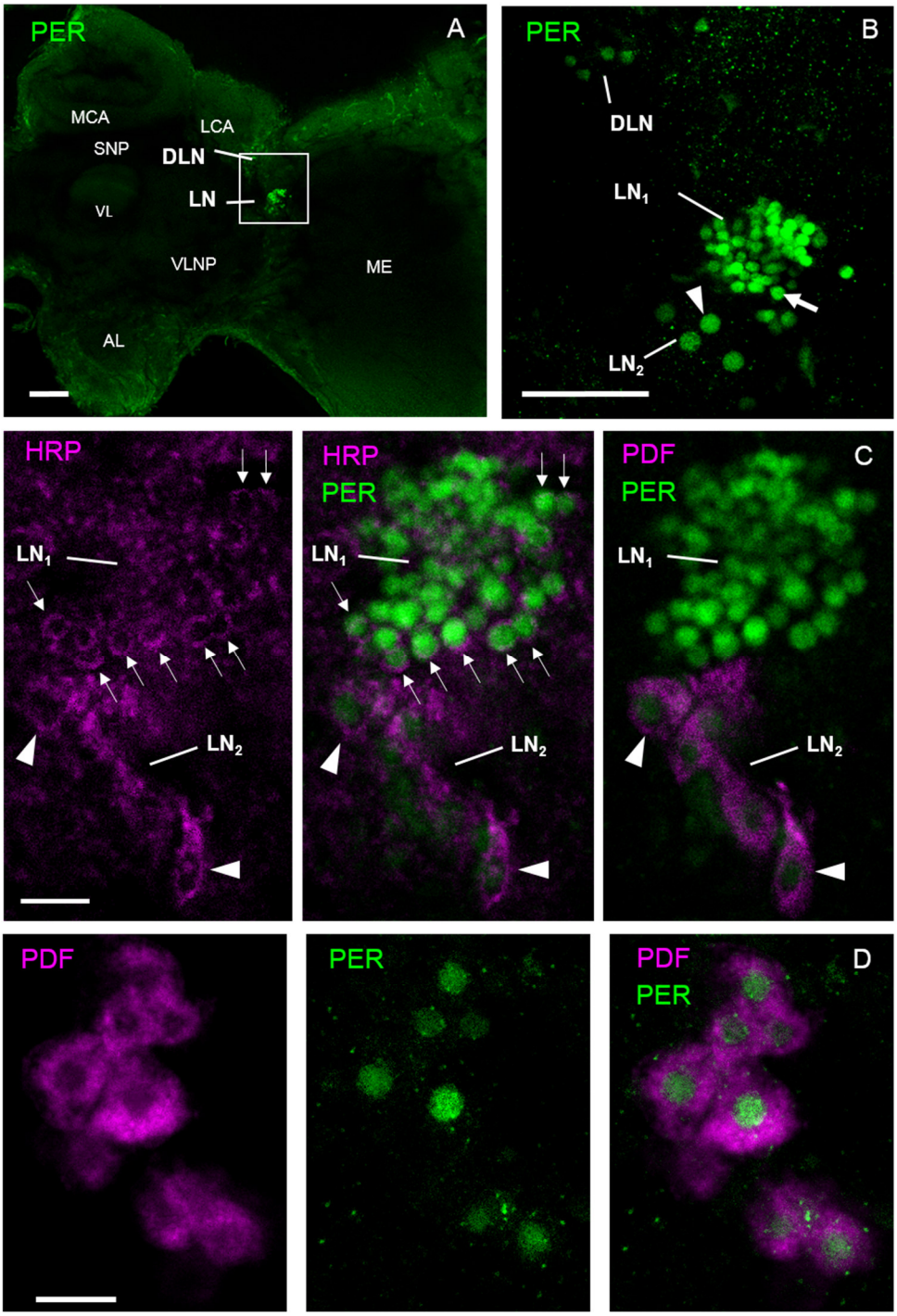

Figure 16: Cell size of PER cells in different clusters.
Projection picture of 60 µm thick agarose section showing PER cell clusters of lateral neurons (LN) and dorso lateral neurons (DLN) **(A)**. **B:** close up picture LNs reveals that some cells are bigger in the lateral cell cluster. **C** and **D:** The PER cells co-labeled with PDF (LN_2) show a bigger nucleus size than the LN_1 cluster (single confocal stack pictures of 150 µm vibratome section **(C)** and of whole mount preparation (**D**) , scale bar: 50 µm). Pictures taken with 10 x objective (numerical aperture: 0.3); distance of z-stacks 2.5 µm. (Adapted from Fuchikawa et al., 2017)

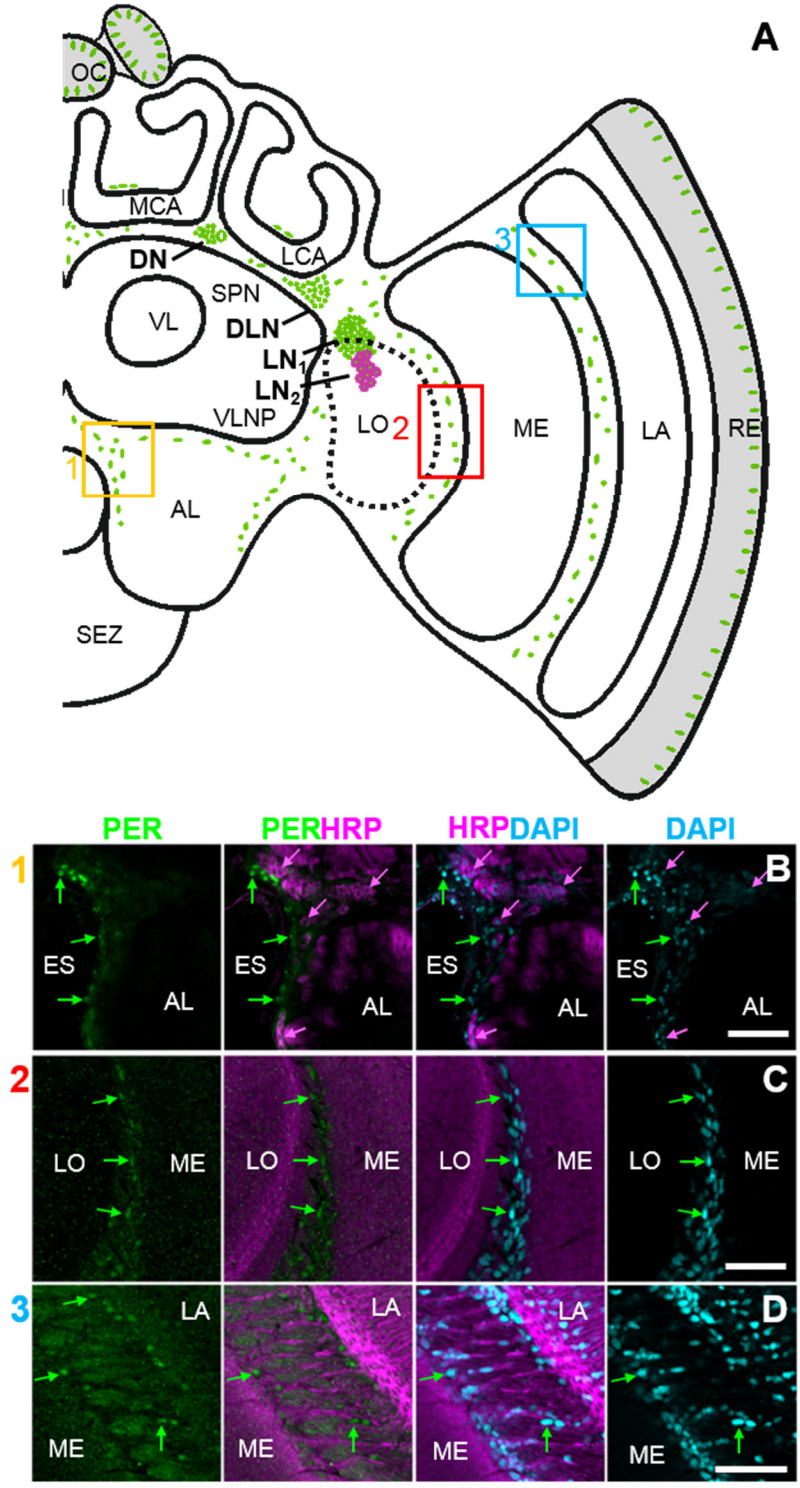

Figure 17: Non-neuronal PER-positive staining in the honey bee brain.
A: same as in Figure 15. Besides the PER neurons there are several other PER-positive signals spread throughout the brain and the optical appendices of the brain, ocelli (OC) and retina (RE). Non neuronal PER staining: **B-D**. PER cells, which are not in clusters and widespread over the brain in areas, like the antennal lobe (**B** area indicated as 1 in **A**), between LO and ME (**C**, area indicated as 2 in **A**) and between ME and lamina (LA) (**D**, area indicated as 3 in **A**) were not double stained with HRP. SPN: superior neuropils, VLNP: ventrolateral neuropils, SEZ: subesophageal zone, MCA: medial calyx, LCA: lateral calyx, VL: vertical lobe. All photos in **(B)-(D)** were taken with a 10 x objective (numerical aperture: 0.3). Please note that only subsets of glia cells are PER positive. Scale bars: 30 µm. (Adapted from Fuchikawa et al., 2017)

Per was further expressed in the optical appendices of the honey bee brain, the retina and the ocelli (**Fig. 18**). I found nuclear *am*PER-positive staining at two different levels of the retina (**Fig. 18 A**). The nuclei at the distal level were clearly labeled by the anti-*am*PER antibody, while those at the proximal level showed only faint staining. There was one PER-positive nucleus per ommatidium at the proximal level, which should correspond to photoreceptor cell 9, whereas in the distal layer there were 8 PER-positive nuclei located at slightly different depths in the ommatidium. These should correspond to the nuclei of photoreceptor cells 1-8. Furthermore, there was nuclear PER staining in cells of a longish shape in the ocelli (**Fig. 18 B**/**C**) and there are PDF cell arborizations reaching into the superior median brain to the base of all three ocelli.

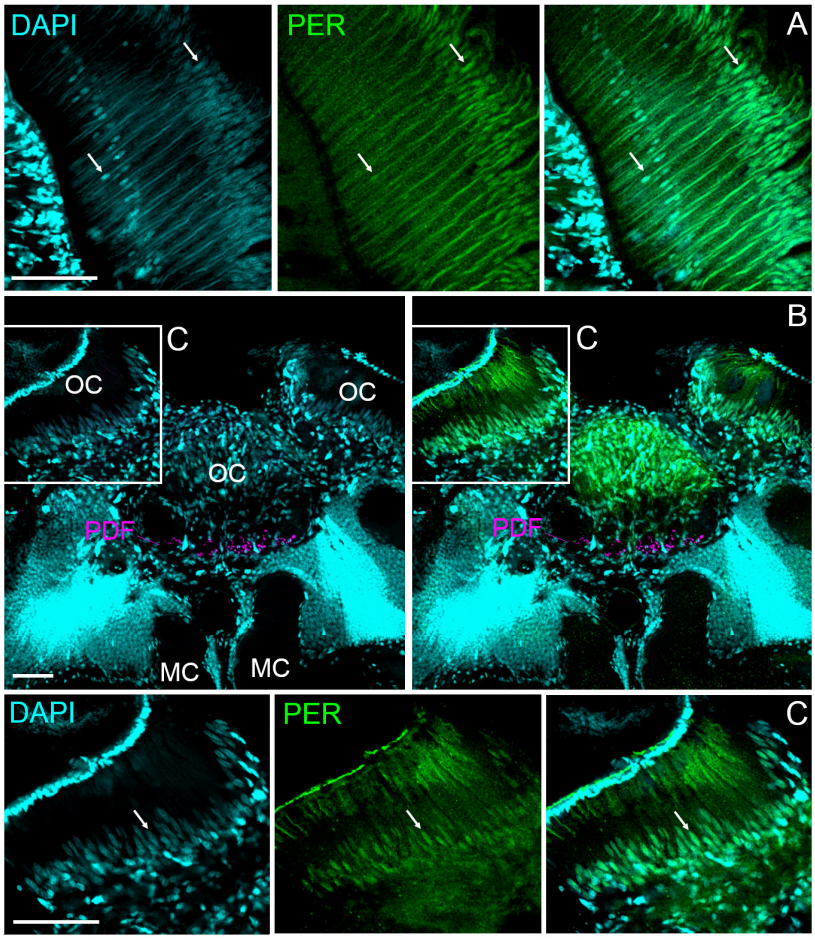

Figure 18: Localization of PER in compound eyes and ocelli.
Projection pictures (2 stacks, stack distance: 2.0 µm; 10 x objective (numerical aperture: 0.3) of nuclear PER staining in the photoreceptor cells in the retina (**A**) and ocelli (**B, C**). DAPI labels the photoreceptor nuclei at a proximal and distal level of the retina (white arrows). Per ommatidium, eight nuclei at the distal level were clearly co-labeled by the anti-*am*PER antibody, the nucleus at the proximal level only faintly. Note that the cornea of the compound eye is detached from the retina due to the whole mount preparation. **(B):** PER, DAPI and PDF (magenta) labeling in the superior median brain and the ocelli (OC). Strong DAPI labeling is present in many glial cells in the ocelli. PDF cell arborizations extend into the base of all three ocelli (at this level of the projection picture only arborizations in the median ocellus can be seen). **(C):** A higher magnification of the left ocellus as indicated in the inset in **B**. MC: medial calyx of the mushroom bodies. Scale bars: 30 µm. (Adapted from Fuchikawa et al., 2017)

Expression pattern of PDF as part of the circadian clock in honey bees (submitted manuscript)

As mentioned in the introduction the expression pattern of PDF in the honey bee brain has already been described in different studies, but without detailed analysis. In another co-laborative project, we show that PDF is part of the circadian clock of bees. Firstly, PDF staining intensity cycles in cell bodies and different parts of the arborization network in brains of forager and nurse bees (PhD thesis Esther Kolbe and manuscript) and secondly locomotor activity rhythms can be time shifted by injection of artificial PDF peptide (manuscript). Thirdly, we analyzed in detail the spatial expression pattern of *pdf* in order to draw further functional conclusions about the role of PDF in the honey bee clock. Therefore, I performed whole mount and section staining (N= 17 brains) of PDF and PER, HRP and DAPI and did 3-D reconstructions of the PDF fiber network and PER cells with Amira. Brains were sampled at ZT 22 (for whole mounts and 60 µm vibratome sections: ambient LD 11:13, for 150 µm vibratome sections: ambient LD 16.5:7.5). In order to fully grasp the arborization network I restored and completed one whole mount PDF reconstruction generated by Esther Kolbe (Kolbe, 2013) and created a 360° view movie (data not shown).

The highly complex PDF network in the honey bee brain is shown in a simplified version in **figure 19**, which displays in the right hemisphere the PER-positive neuron clusters DN, DLN, LN_1 (green) and the PDF and PER positive neurons of the LN_2 cluster (green and magenta) as in the chapter above. In the left hemisphere there are additionally depicted the arborizations (magenta) of the PDF neurons (LN_2) throughout the brain and areas with PER-positive staining in glia cells (green). The arborization network reaches almost every part of the brain, innervating the LA and ME in the optic lobes as well as many neuropils in the protocerebum. PDF neurites reach to the OC, AL and surround the esophagus in the esophageal foramen of the SEZ. Furthermore, a dense PDF network closely encloses the mushroom bodies and PDF fibers cross the brain in at least seven commissures (blue numbers). Commissure 1 (anterior optic commissure) and 7 are the most anterior positioned and 4 (posterior optic commissure) is the most posterior. In between are two double commissures laying dorso anterior (2,3) and ventrally (5,6) of the central complex marking the top and the bottom of a triangle formation in the network (CX is not shown in **fig. 19**, see also **fig. 23**). To be exact, the triangle formation in the middle of the brain consists of three triangles: Two pointed to the dorsal brain with commissures 5 and 6 as base side and a third triangle pointing ventrally and commissures 2 and 3 build the base side. The scheme highlights only major fiber bundles, while very thin arborizations are excluded in order to present a general overview. In the projection of the network to one plane, some fiber bundles cannot be drawn in the absolute correct position, but they are still recognizable in the confocal pictures following.

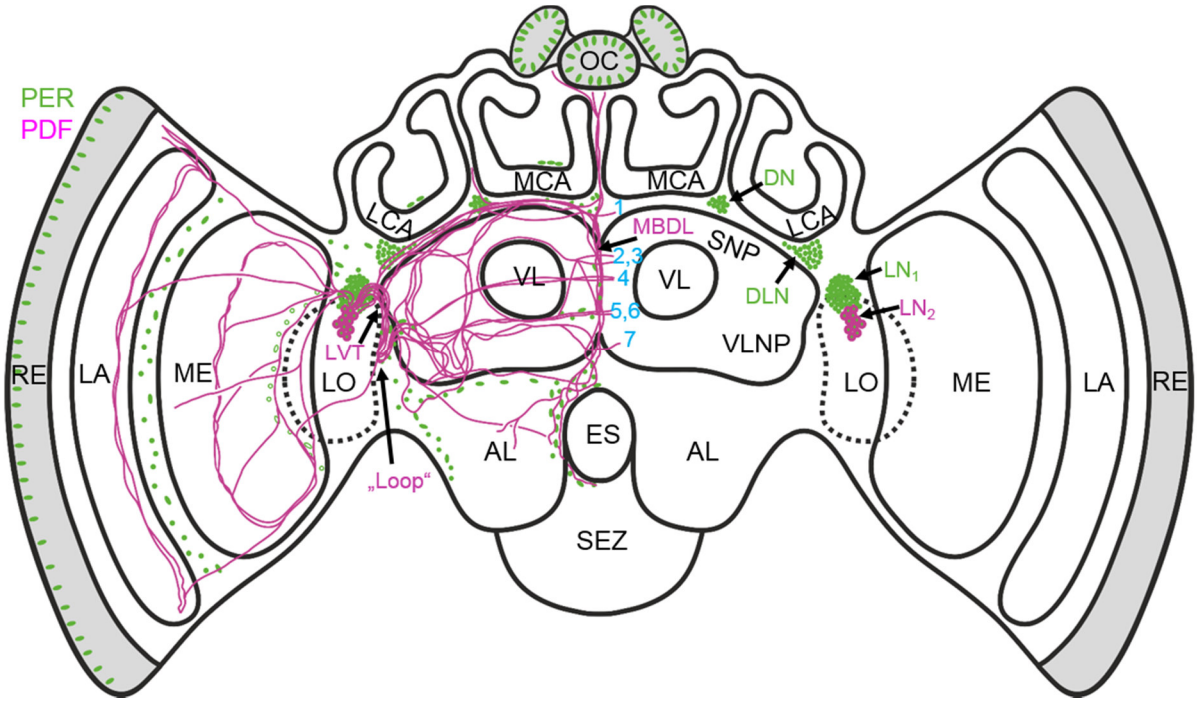

Figure 19: Scheme of PER- and PDF-positive cells in the brain of the honey bee *Apis mellifera*. Nuclei of PER positive cells (green) and somata of PDF positive neurons (magenta) in the honey bee brain. In the left brain hemisphere, additionally the arborisations of the LN_2 neurons (magenta) are depicted together with the nuclei of PER positive glia and photoreceptor cells (green). A major fiber bundle (LVT = lobula valley tract) is running dorso-ventrally along the proximal rim of the LO (lobula) and forming a loop, from which most of the neurites branch in the protocerebrum and a thick fiber bundle builds the POC (posterior optic commissure). Blue numbers mark the main commissures: 1: anterior optic commissure; 2,3: double commissure dorsally of the central complex; 4: POC; 5,6: double commissure ventrally of the central complex; 7: ventral anterior commissure. LN_1: lateral neurons 1, LN_2: lateral neurons 2, DLN: dorsolateral neurons, DN: dorsal neurons. OC: ocelli, LCA, MCA: lateral and medial calyces of the mushroombodies, VL: ventral lobe of the mushroom bodies, AL: antennal lobe, ES: esophagus, SEZ: subesophageal zone, ME: medulla, LA: lamina, RE: retina, SNP: superior neuropils of the protocerebrum, VLNP: ventrolateral neuropils of the protocerebrum, MBDL: median bundle

The LN_2 neurons are located adjacent to the ME and anterior of the LO (**Fig. 20**). They all project dorsally to the LN_1 cluster and divide then into fibers running to the OL and the protocerebum. A small fiber bundle innervates the ME, especially the serpentine layer between proximal (pME) and distal medulla (dME) (**Fig. 20 B**, arrowhead). Some other neurites run dorsally of the ME into the direction of the dorsal rim area (DRA) (**Fig. 20 B**, double arrowhead), but the dorsal rim area of the medulla (MEDRA) seems not to be innervated. The most dense fiber bundles nevertheless are not to be found in the area which might contribute to an accessory medulla (AME), but are located dorsally of the LN_1 cluster (**Fig. 20 B**, red arrow). We called this high density fiber network ALO (anterio-lobular PDF hub) according to its location. From the ALO fibers run into the ME (and putative AME) and also into the direction of the protocerebrum. Large tracts, as the anterior optic tract (AOT) usually contain the nuclei of glia cells, whereas the small neuropil structure (putative AME) adjacent to the ME is basically free of nuclei (**Fig. 20 D**). In figure 22 only the beginning of the neurites directed to the protocerebrum are shown, but they continue mainly in the lobula valley tract (LVT) and a dense fiber bundle between the

calyces of the mushroom bodies and the SNP, which continues into the median bundle and the anterior optic commissure (see also **fig. 19, 22, 24, 25**).

PDF is not only co-expressed in a subset of the PER neurons, but it is also in close proximity to PER-positive glia cells in many areas throughout the brain. Especially close are the projections to the LN_1 neurons. The LN_2 project dorsal and posterior through the LN_1 cluster and thereby connect many of the cells (**Fig. 21**). From here, the arborizations grow out laterally to the medulla and the protocerebrum, where the fiber bundle separates into fibers running to the dorsal and ventral areas. The reconstruction is only for the anterior part of a whole mount honey bee brain and therefore most of the thick fiber bundle building the LVT is not shown (see also **fig. 19, 22, 23, 24**). On the other hand, the PDF fibers pass closely by only a few of the DLN neurons. Most of the cells in this widespread cluster are in a distance from PDF stained structures (**Fig. 21C**), but the DN cluster is again quite close to the PDF fibers (Fig. 21, 24B).

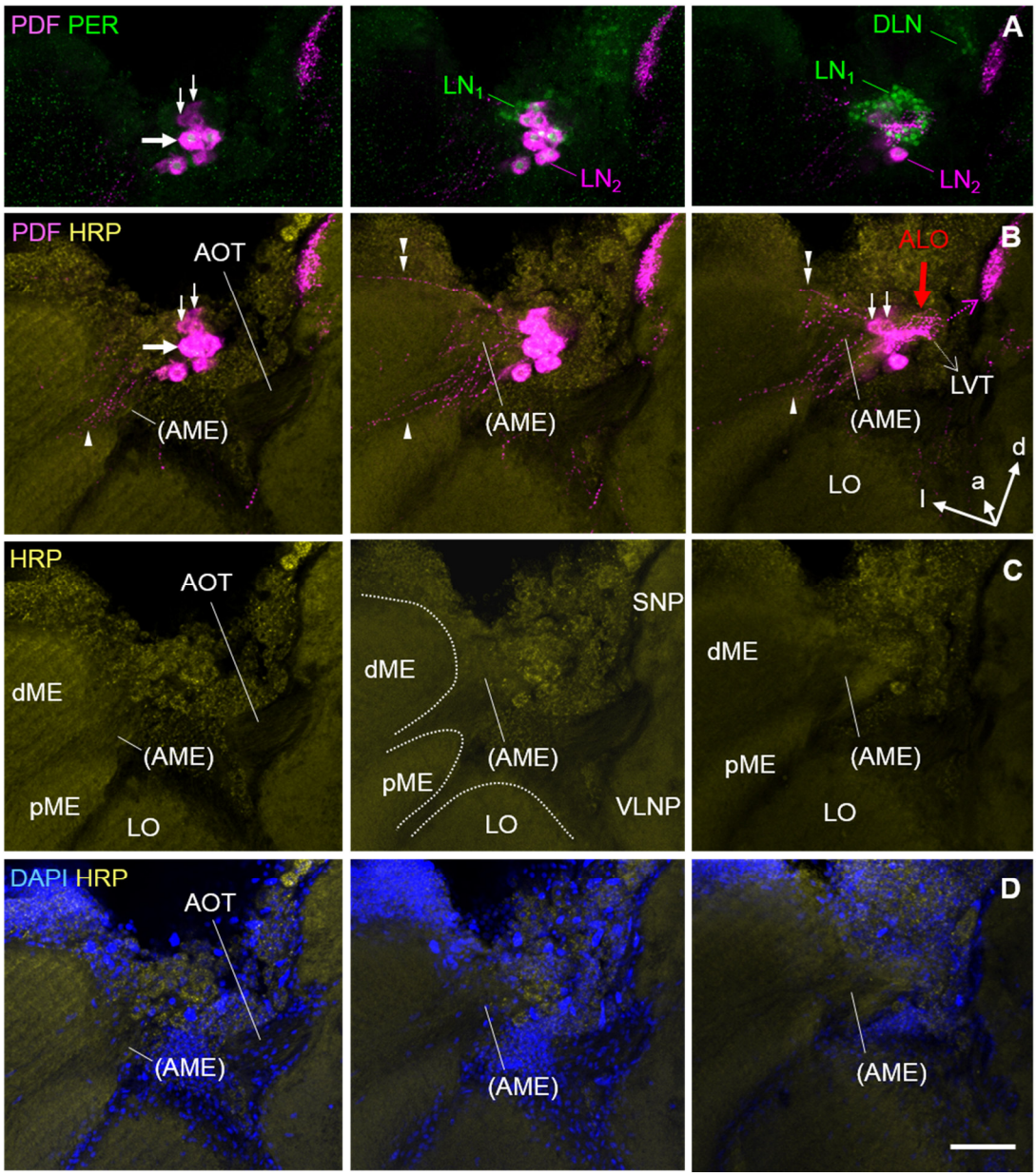

Figure 20: The putative accessory medulla (AME) and the high density anterio-lobular PDF hub (ALO).
Overlays of 6 confocal stacks, each, in the anterior (left), medial (middle) and posterior (right) area of the left accessory medulla (AME) of a 60 µm thick semi-frontal vibratome section. Note that this section is tilted in the anterior-posterior as well as in the left-right plane to visualize the serpentine layer of the medulla and the putative AME. The section is labelled with anti-PER, anti-PDF, anti-HRP and DAPI. **A:** PER and PDF labelling in the dorsal region of the pictures. The PDF positive neurites are hard to see, because PDF labelling intensity is reduced. The large arrow points to the largest PDF neuron, the small arrows to the smallest PDF neurons. **B:** PDF labelling at higher intensity revealing the arborizations of the LN_2 together with HRP labelling. **C:** HRP visualizes the major neuropil regions (plus the somata of neurons): dME distal medulla, pME proximal medulla, LO lobula, putative accessory medulla (AME), SNP superior neuropils, VLNP ventrolateral neuropils). The neuropils are free of somata and nuclei as can be seen by the combination of DAPI and HRP labelling (**D**). The PDF neurons send sparse neurites into a small neuropil structure reminiscent of the AME and from there into the serpentine layer of the medulla (arrowhead) and to a minor degree into the most distal layer of the medulla (double arrowhead). Nevertheless, mainly they send fibres with dense varicosities onto the surface of the lobula (ALO, red arrow) and from there to the superior (SNP) (magenta broken arrow). The lobula valley tract (LVT) originates from the dense PDF fiber network ALO and projects posteriorly and ventrally along the inner surface of the lobula. Please note that the AOT is free of PDF fibers. Scale bar: 50 µm; orientation arrows: l: lateral, a: anterior, d: dorsal

In case of the PER glia cells this is quite similar. Most of them are just adjacent to PDF fibers (**Fig. 21, 22**). In **figure 22**, are some examples for the spatial relationship of PER positive glia and PDF fibers. The differentiation of neurons and non-neuronal cells (putative glia) was done with anti-HRP co-labeling. HRP stains specifically only the somata of neurons. Since I have already shown in the last chapter detailed HRP staining of the PER positive neurons the overview pictures A-C are shown without the anti-HRP labeling. One detail picture of PER/HRP/PDF triple staining in the SNP at the region where the DN cluster lies is displayed in the supplemental figure 3 (**Fig. S3**). The DN cluster is positioned dorsally of the PDF fibers that run into the SNP and in a few case single PDF fibers pass by the DNs when reaching from anterior to posterior between MCA and LCA of the mushroom bodies. The PDF neurites surrounding the VL often are in close proximity to PER-positive glia cells of mainly longish shape (white arrows in **fig. 22 A**/**B** and **S3**). The blue arrows in **figure 22 B** and **C** point to the two most anterior positioned commissures. In the PI (**Fig. 22 D**), the PER glia cells are not as closely to the PDF fibers as in the other examples. Along the MBDL (**Fig. 22 E**), the LVT and POC (**Fig. 22 F**) and at the rim of the AL and the esophageal foramen (**Fig. 22 G-I**) PER glia are in very close proximity to the PDF neurites. The last 3 pictures show the same area from anterior to posterior, indicating that the AL are innervated from posterior to anterior by PDF fibers originating from the posterior ventral protocerebrum around the ventral end of the MBDL, but the PDF fibers seem not to reach into the glomeruli of the AL. One single fiber connects the LVT to the esophageal foramen directly crossing through the SEZ (**Fig. 22 I**, magenta arrow). Other examples for glia cells in close proximity to PDF fibers are in the optic lobe, especially in the DRA, the proximal layer of the LA and between LO and pME (**Fig. 23 A,B** and **C** white arrow). In the DRA, dense PDF and PER staining can be found as well as in the proximal LA, while in the serpentine layer of the ME between dME and pME there are only PDF neurites and between ME and LO there are many PER positive glia, but only very few PDF fibers. Many PER glia cells are located around the central body (**Fig. 24**) of which some seem to be in contact to PDF neurites running through the MAL (medial accessory lobe of the central complex) towards the MBU (medial bulb of the lateral complex) (white arrow).

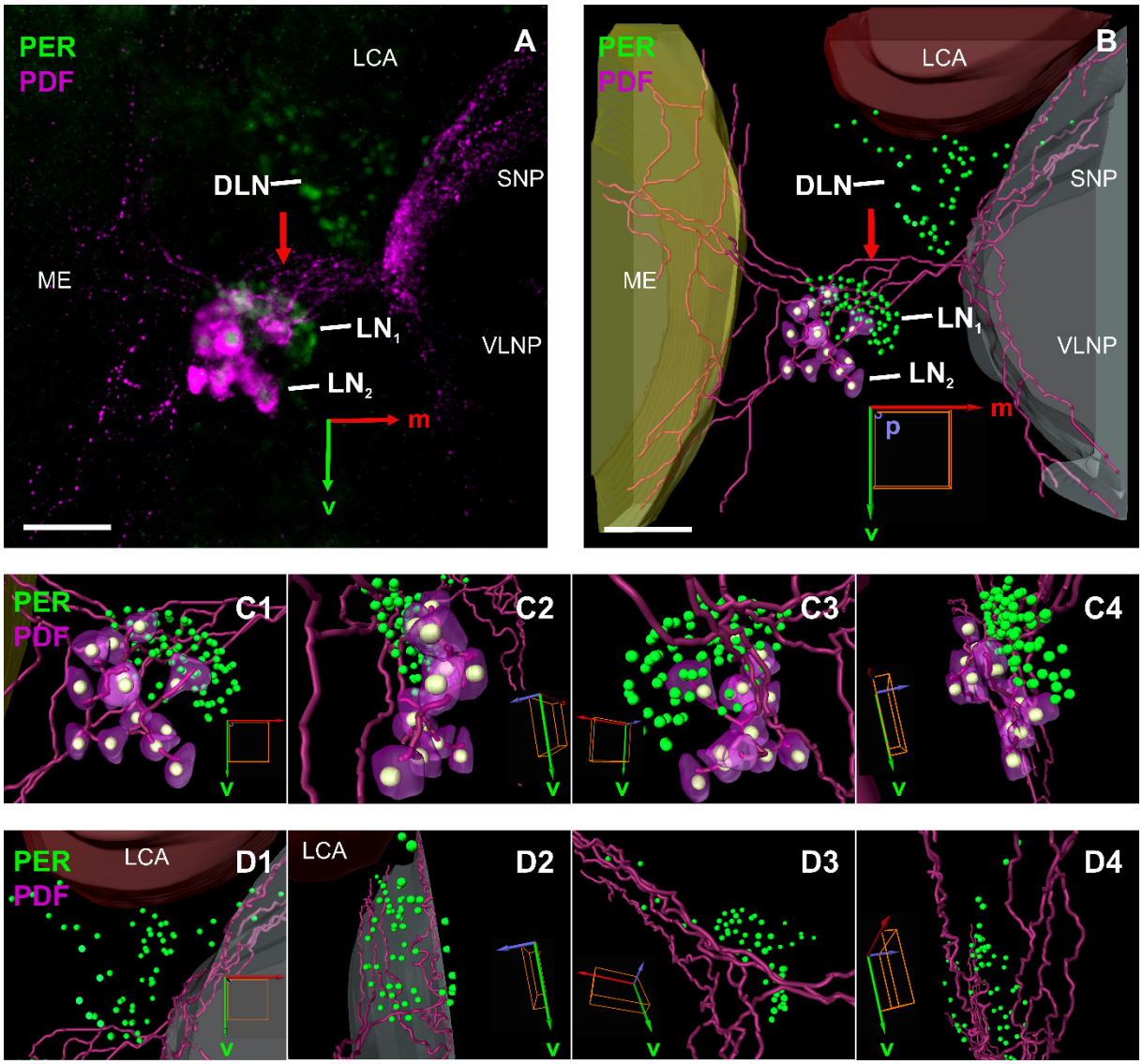

Figure 21: Vicinity of the PER-positive LN_1 and DLN clusters to PDF-positive fibers originating from the LN_2 cluster.
A: Projection picture of several layers scanned though the left lateral part of a whole mount honey bee brain and relevant reconstruction of this part in Amira (**B**). PDF (magenta) is present in the cytoplasm of the LN_2 cluster and labels additionally its neurites, whereas PER (green) labels the nuclei of the three cell clusters (DLN, LN_1 and LN_2). The nuclei of the LN_2 cluster are shown in white in the reconstruction. The PDF neurites running towards the medulla (ME) are less dense than the dense PDF network dorsally of the LN_1 (anterio-lobular PDF hub (ALO), red arrow), from which fibers run towards the superior (SNP) and ventrolateral neuropils (VLNP). The LVT is not yet visible at this anterior level. (**C**) Larger magnification of the reconstructed LN and (**C**) DLN clusters viewed from anterior, lateral, posterior and medial, respectively. Note that PDF-positive neurites from the LN_2 are in close proximity to the LN_1, while the fibers running into the SNP are only close to a few of the cells in the DLN cluster. Scale bars: 50 µm. LCA: lateral calyx; orientation arrows: m: medial, p: posterior, v: ventral

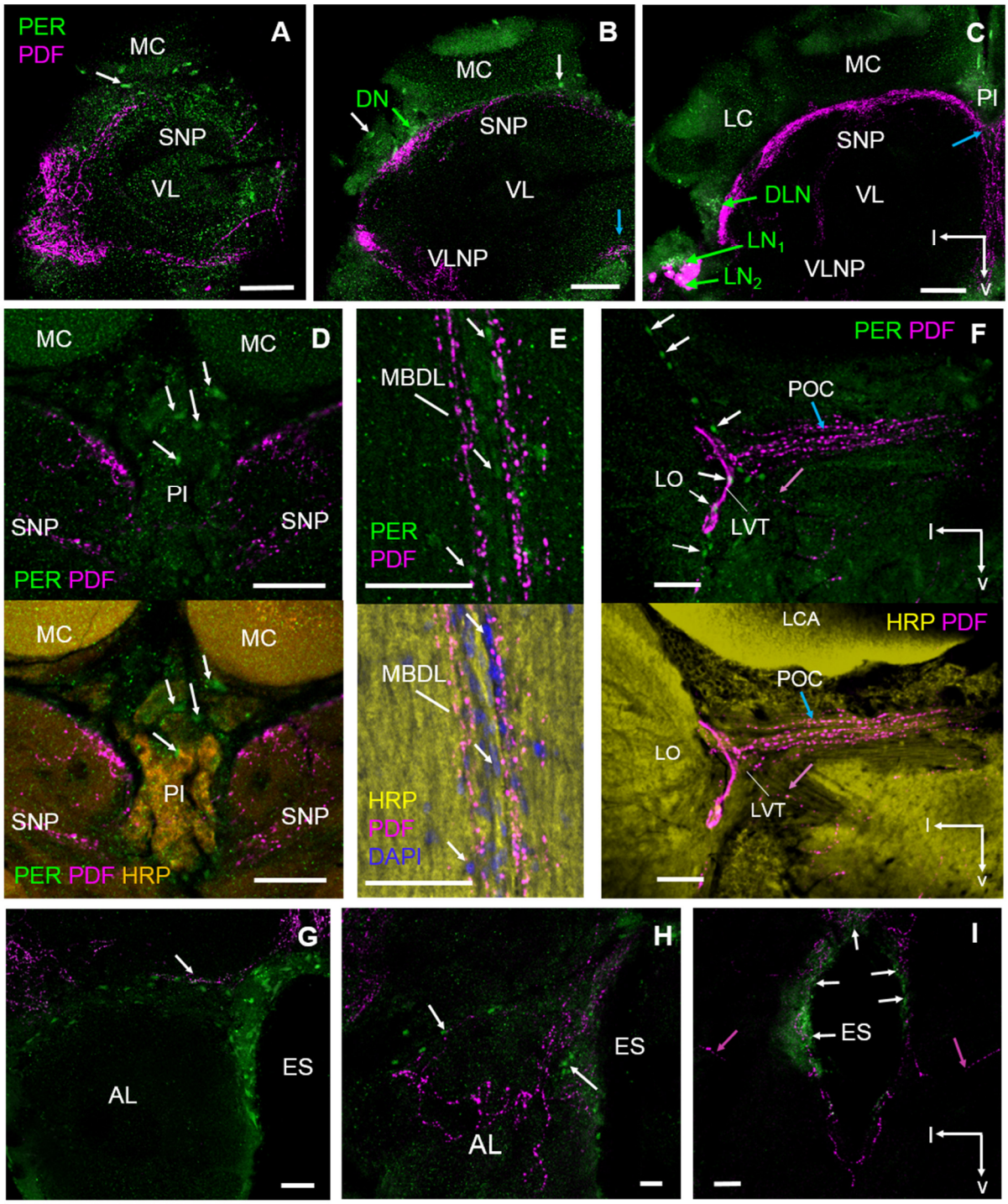

Figure 22: Examples for close vicinity of PDF fibers and PER glia.
Projection pictures of a whole mount double staining with anti-PER (green) and anti-PDF (magenta) antibodies from anterior to posterior (**A-C**). Pictures **D-I** display the spatial relationship of PER positive glia (green) and PDF neurites (magenta) in detail pictures. Besides the PER neuronal clusters LN_1, LN_2, DLN and DN (green arrows) the PER positive glia are often in close vicinity to the PDF neurites (white arrows), i.e. along the median bundle (MBDL) (**E**), the LVT (lobula valley tract) and the POC (posterior optic commissure) (**F**), the AL (antennal lobe) (**G, I**) and the esophageal foramen of the ES (esophagus) (**I**). Anti-HRP staining (yellow) marks the neuropils and somata of neurons, while somata of putative glia cells with nuclear PER staining show no HRP staining. Blue arrows mark the two anterior commissures (**B, C**) and the POC (**F**) as described in **fig. 19**. Magenta arrows (**F**) mark a medial PDF fiber tract originating closely to the loop and running towards the triangle network at the central complex. Scale bars: 100 µm in **A-C** and 50 µm in **D-I**. Orientation arrows: l: lateral, v: ventral. MC: medial calyx of the mushroom bodies, SNP: superior neuropils, VLNP: ventrolateral neuropils, LO: lobula.

Apart from PDF fibers in the optic lobe (**Fig. 23 A-C**) I found PDF staining in or at least very close to neuropils that are involved in the polarized light input pathway in the protocerebrum (**Fig. 23 D, E, H**). In the anterior input pathway, the AOTu plays a central role and PDF neurites densely surround this neuropil dorsally and posteriorly. In the frontal sections, it is hard to judge how far the network reaches from posterior, but some PDF fibers seem to reach into the dorsal rim of the AOTu (**Fig. 23D**, white arrow). Moreover, single PDF neurites innervate the edges of the POTu (posterior optic tubercle) (**Fig. 23 E** / **H**) which lie just posterior of the PB (protocerebral bridge) and extend to a region posterior of the POC. Also in this area, PDF fibers arising from the POC run very close to the boundaries of the neuropil (**Fig. 23 H**). Other PDF fibers from the POC branch out into the direction of the ocelli (**Fig. 23F**, white arrows). There are some other PDF neurites, which run anterior into the direction of the medial ocellus, but these seem to arise from the double commissure ventrally of the central complex (CX) and not from the POC (**Fig. 23 G**, white arrow).

As mentioned above the dorsal and ventral double commissures mark the edges of the CX (**Fig. 24**, blue arrowheads: double commissure 2,3; blue arrows: double commissure 5,6). Commissure 6 (**Fig. 24 B**/**D**, blue arrow) lies posterior of commissure 5 (**Fig. 24 A**, blue arrow) and densely innervates the MAL. Further PDF neurites reach to the edges of the MBU, the NO (noduli of the CX) and the LAL (lateral accessory lobe of the CX) or are in close proximity to these structures (**Fig. 24B**/**D**, white arrow). The ventral double commissure represents the ventral connection of the triangle formation of the PDF fibers in the central brain, while the other sides of this triangle pass laterally by the CB and posterior of the ML (medial lobe of the mushroom bodies). The tip of the triangle structure is connected to the dorsal double commissure further anterior. The fibers of the triangle originate mainly from a dense network located in the anterior brain (posterior of the AOTu) but also from fibers directly from the LVT running medially towards the central brain (**Fig. 24C**), fibers in the POC and in a more ventral medial tract that seems to start close to the loop (**Fig. 22F**, magenta arrow). PDF arborizations are in general very varicose, like seen for example in the POC and the medial tract, whereas the LVT contains virtually no varicosities. The LVT originates from a very dense fiber tract dorso medial of the PDF somata it runs posterior and then along the side of the LO to the ventral brain. There it forms a characteristic anterior-posterior loop before the fibers run into the medial protocerebrum and the POC. Whether these fibers originate from the LVT or from the contralateral hemisphere and enter the LVT, or both, could not be determined. The same is true for the direction of projections in the optical lobe: fibers that leave the serpentine layer of the medulla and run over the posterior surface of the lobula leave or enter the LVT. It seems that one particular neuropil is a special case in the PDF arborization pattern. Although the PDF neurites never innervate the mushroom bodies they are in very close proximity and surround the neuropil in a dense network around the VL, ML, PED and also reach close to the calyces (**Fig. 25**, magenta arrows).

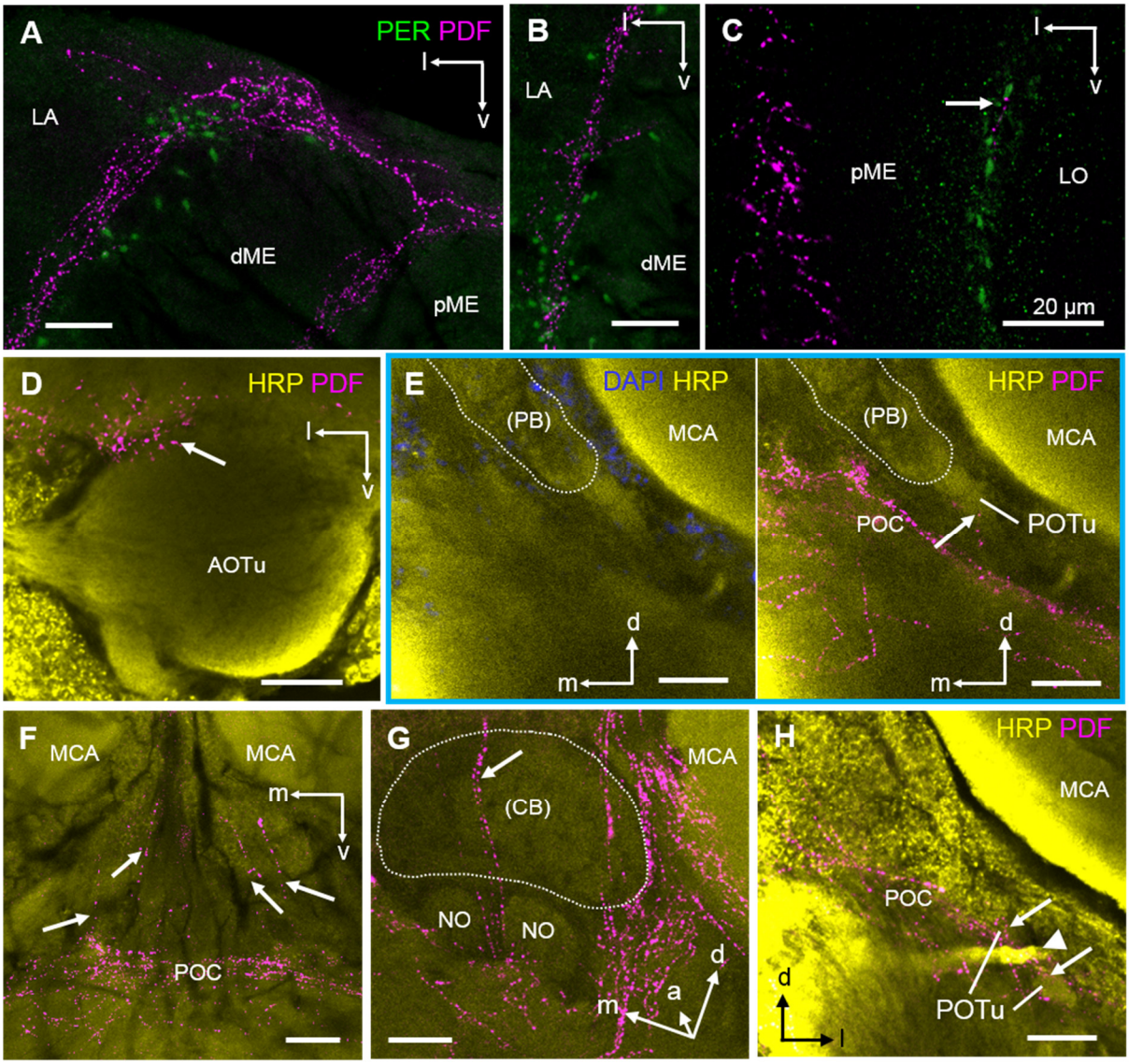

Figure 23: PDF and PER cells along the light and polarized light input pathways.
Projection pictures of PER (green) and PDF (magenta) double staining in the dorsal rim area of the lamina (LA) (**A**), along the proximal layer of the LA (**B**) and between the pME (proximal medulla) and the lobula (LO) (**C**). Note that there is a dense innervation of the serpentine layer of the medulla between dME (distal medulla) and pME but no PER cells, while between the pME and the LO the PDF innervation is sparse (white arrow) and there are mainly PER glia. PDF neurites (white arrows) further reach into the rim of the AOTu (**D**) and the POTu (**E, H**), which are part of the anterior and posterior polarized light input pathway respectively. Several PDF fibers originating from the POC (posterior optic commissure) run dorsally towards the ocelli (**F**, white arrows). Another fiber bundle branches of the double commissure ventrally of the CB (central body), runs between the NO (noduli of the central complex) and continues anterior of the PB (protocerebral bridge) and posterior of the CB towards the medial ocellus (**G**, white arrow). Only the edges of the PB and the CB are displayed in the pictures wherefore I added dotted lines to better outline the neuropils. Neuropils are stained with anti-HRP antibody (yellow) and DAPI staining shows a lack of nuclei in the neuropils to mark these additionally. MCA: medial calyx of the mushroom bodies. Scale bar: 50 µm except for **C** (20 µm). Orientation arrows: m: medial, a: anterior, d: dorsal, l: lateral, v: ventral

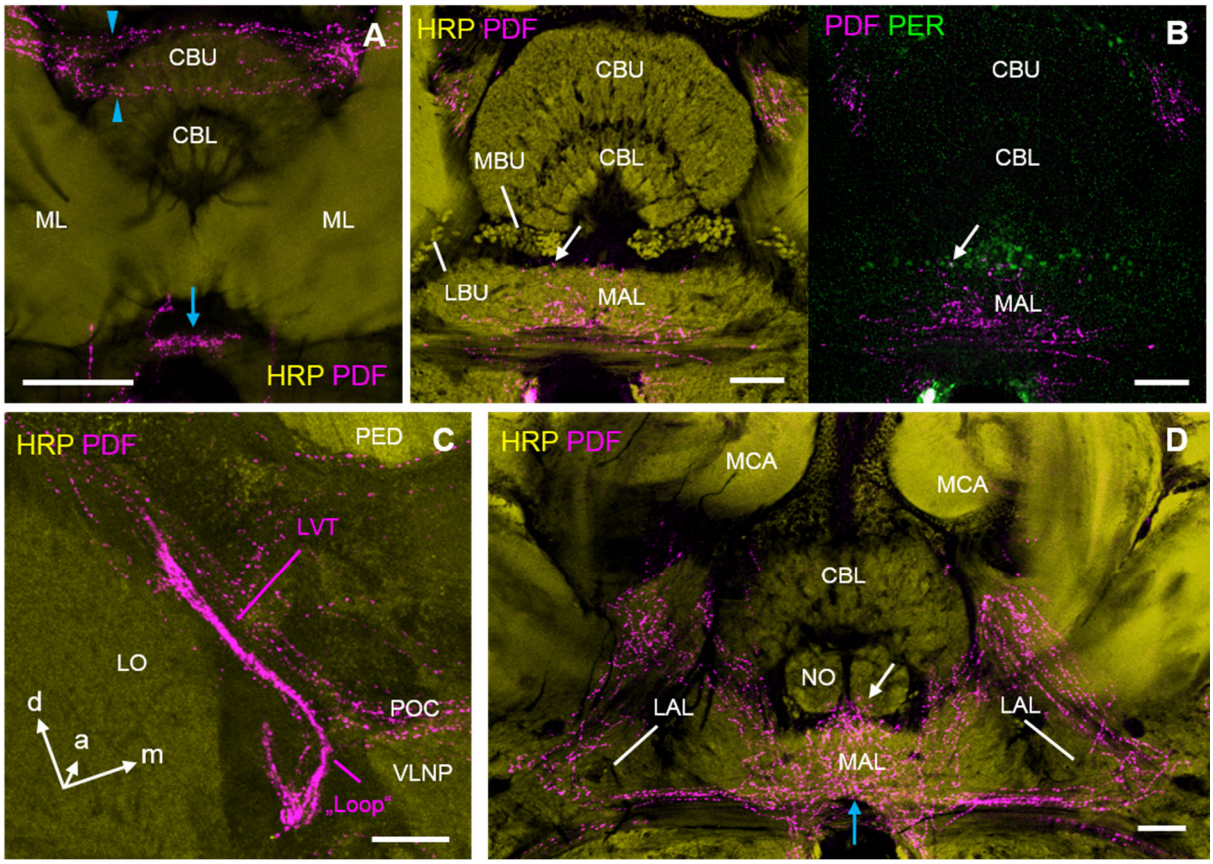

Figure 24: PDF network in the central brain.
A, B, D: PDF fibers (magenta) form a triangle network around the central complex. **A:** picture of area of the ML (medial lobes of the mushroom bodies) and the anterior part of the CX displays the dorsal double commissures (blue arrowheads) at the top of the triangle structure and the anterior part of the ventral double commissure (blue arrow). **B:** Medial area of the CX shows PDF fibers along the sides of the CBU (upper division of the central body) and dense innervation of the MAL (medial accessory lobe of the central complex). Only few PDF fibers seem to reach into the rim of the MBU (medial bulb of the lateral complex) and connect PER glia cells (green) located close to them (white arrow). In a projection picture of the medial to posterior part of the CX (**D**) the whole triangle structure is represented, indicating also PDF projections reaching the edges or close to the LAL (lateral accessory lobe) and the NO (noduli of the central complex) (white arrow). **C:** The LVT (lobula valley tract) in the lateral brain with its characteristic anterior-posterior loop formation is the origin of the dense PDF network in the central brain. Anti-HRP (yellow) staining marks the neuropils. CBL: lower division of the central complex, LBU: lateral bulb of the lateral complex, PED: pedunculus of the mushroom bodies, MCA: medial calyx of the mushroom bodies, VLNP: ventro lateral neuropils of the protocerebrum. Scale bar: 50 µm. Orientation arrows: d: dorsal, a: anterior, m: medial.

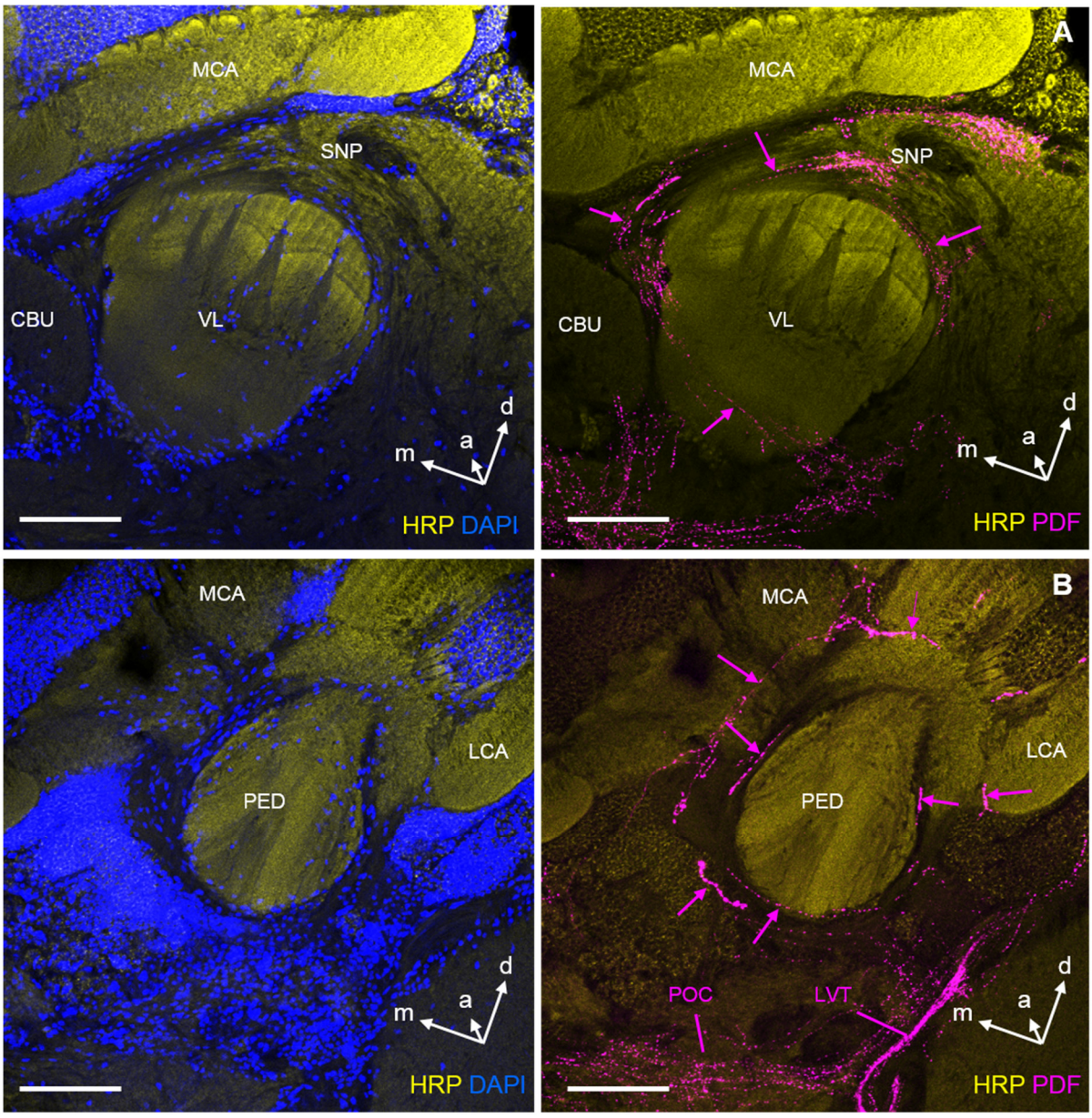

Figure 25: Dense PDF network surrounding the mushroom bodies.
PDF fibers (magenta) running close to the edges of the mushroom bodies and surround them in a dense network (magenta arrows) around the VL (vertical lobe of the mushroom bodies) (**A**), the medial lobe and the PED (pedunculus of the mushroom bodies) (**B**). Furthermore, the thick PDF fiber bundles dorsally of the SNP (superior neuropils of the protocerebrum) are in close proximity to the MCA and LCA (medial and lateral calyces of the mushroom bodies). Neuropils are marked by anti-HRP staining (yellow) and the lack of DAPI staining (blue). CBU: upper division of the central complex, POC: posterior optic commissure, LVT: lobula valley tract. Scale bar: 50 µm. Orientation arrows: m: medial, a: anterior, d: dorsal

Neuroanatomical localization of PDF and PER in *Osmia*

In the solitary bees, the neuroanatomical organization of the circadian clock seems to be quite similar to the honey bee clock, at least regarding the two investigated components PDF and PER. I double stained 11 brains of *A. mellifera* foragers (ZT22), 17 brains of *O. bicornis* (age 1 week, sampling time points: ZT 2, 6, 14, 18, 22) and 3 brains of *O. cornuta* (ZT22) in whole mount preparations with anti-PDH and anti-PER antibodies. Experiments where performed during spring and summer and the entrainment in climate chambers was LD 16:08 (*O. bicornis*) and LD 14:10 for *O. cornuta*, while honey bees where entrained in their hive (natural light conditions: LD 16.5:7.5 and 12:12 in two different trials) and sampled during the night in an incubator (see chapter 2.2.1.). All sampled bees were female to exclude potential sex differences in the expression pattern of the clock components. The PDF network appears in the overview very similar to the one in honey bees, with 13.8 somata of the PDF neurons (LN_2 cluster) located adjacent to the ME and the lateral dorsal protocerebrum. The neurites reach into almost every brain area in fiber bundles structured like in the *A. mellifera* brain (for details see **fig. 26**). Nevertheless, there are some differences: Firstly, some fiber bundles seem to consist of less neurites or be less compact than in the honey bee, which is most obvious in the LVT and the POC (**Fig. 26 D**/**E**). This seems to be the case also in the serpentine layer of the ME, but the position of the optic lobe is slightly shifted in the picture and therefore a comparison in a 3-D reconstruction would be preferable to this comparison in projection pictures. Another very striking difference is in the LA, where in the dorsal half the arborizations extent and build a densely innervated structure (⌀ 27-30 µm), which is missing in the honey bee LA (**Fig. 26 E**, black arrow).

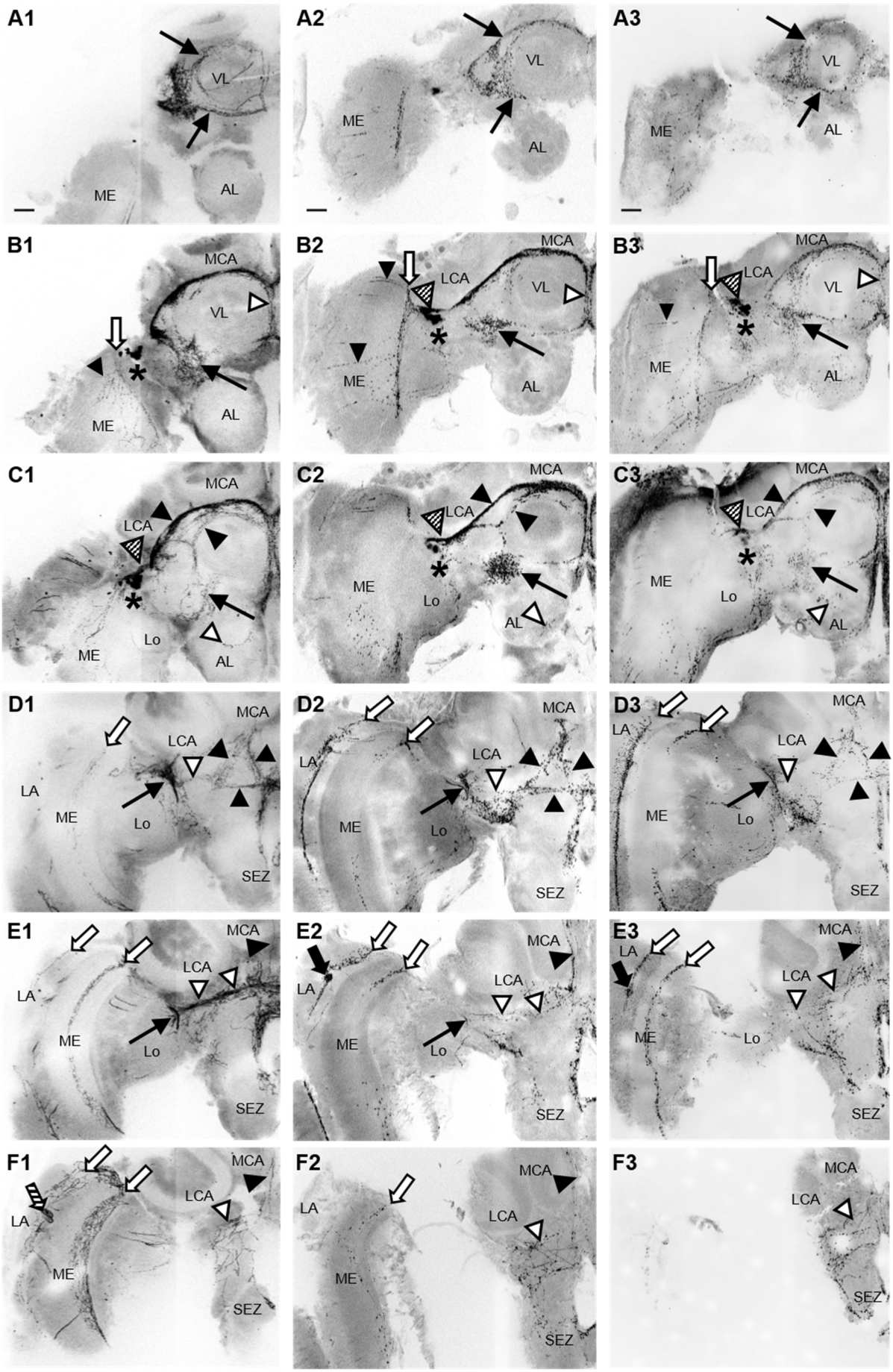
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Figure 26: Comparison of the PDF network in social and solitary bees.
Projection pictures of PDF staining in whole mount preparations of *A. mellifera* (1), *O. bicornis* (2) and *O. cornuta* (3). From A to F projection pictures (10-33 stacks) display corresponding brain areas in the different bee species (10 x objective; numerical aperture: 0.30, resolution: 1024x1024, z-step size: 4 µm). Note that the brains are not exactly in the same position due to restrictions in the mounting procedure, wherefore I combined the projection picture from different stack numbers in order to present the corresponding structures in the PDF network. From the cell bodies (asterisk in B/C) fibers run dorso-posterior and intermingle in a dense fiber network I called ALO (anterio-lobular PDF hub, striped arrowhead). From this network, fibers continue into the optic lobe and central brain. Two fiber bundles, one dorsally one ventrally of the protocerebrum, run to the medial brain building either the two anterior commissures (anterior optic commissure and ventral commissure) or run into the median bundle (white arrowhead B). One sparse fiber bundle innervates the proximal medulla (white arrow B) and sends single fibers into the fiber fan on the anterior rim of the medulla (black arrowheads B). While the dorsal network seems to consist of different thick fiber bundles splitting and reuniting to a network in the superior neuropils of the protocerebrum (black arrowheads C) the ventral fibers create a dense network posterior of the AOTu (black arrow B/C). The AL is innervated (white arrowhead C) from posterior fiber bundles in the medial brain. The dorsal and ventral fiber bundles mentioned above as well as a medial fiber bundle (white arrowhead D) and the POC (white arrowhead E) arise from the LVT (black arrow D/E). The characteristic triangle structure (black arrowheads D) around the central complex is formed by PDF neurites originating from the dense anterior ventral network and the medial fiber tract and the top of the triangle is connected to the dorsal fiber bundle. D, E and F display innervation of the proximal layer of the LA (white arrow) and the serpentine layer of the ME (white arrow). Further neurites branching of the POC towards the ocelli (black arrowhead E, F) are displayed. In the *Osmia* brains, there is an area in the more dorsally located LA, which shows a small, but dense network compared to the rest of the fiber bundles in the LA (black arrow E). This structure is missing in the honey bee (striped arrow in F is not the corresponding structure, but dirt on the surface of the preparation). In the posterior protocerebrum, there is a loose network of single PDF fibers (white arrowhead F). Scale 100 µm.

Unfortunately, the anti-*am*PER antibody recognized the *osmia*PER not as nicely as the *am*PER protein, wherefore there was only faint PER staining and very high background noise in the *Osmia* preparations. Best results were achieved for the whole mount preparations of *O. cornuta*, in which I identified the same PER positive neuronal clusters as in the honey bee (**Fig. 27**) and named them analogously. One cluster (dorsal neurons: DN, **fig. 27 B**) is positioned in the dorsal, anterior brain between the two calyces of the mushroom bodies and the SNP. Another PER neuron cluster lies adjacent to the SNP in the dorsal lateral brain (dorso lateral neurons: DLN, **fig. 27 C**) and in the lateral brain the two lateral clusters (lateral neurons 1 and 2: LN_1, LN_2) were identified. Like in the honey bee brain in the LN_2 cluster *per* and *pdf* are co-expressed and PER staining is only nuclear while PDF can be found only in the somata of the neurons (**Fig. 27 D-F**). I counted the cell number for the different PER-positive cells in the clusters in brains sampled at ZT 22. At this time point *per* displayed the highest expression. There were approximately 13 neurons in the DN cluster and 12 neurons in the DLN cluster, which I could only detect in brains of *O. cornuta*. The biggest cluster was the LN_1 cluster with 25 neurons (in *O. cornuta* and *O. bicornis*) and the LN_2 cluster located anterior and ventrally of the LN_2 had a number of 16 cells (in *O. cornuta* and *O. bicornis*). There were a few PER-positive cells close to the LN_2 neurons, but located ventrally of them. This I rarely observed in *A. mellifera* brains. Nevertheless, I suggest these cells might belong to the LN_1 neurons, because they do not show pdf expression and are approximately the same size as the LN_1 (LN_2 neurons are bigger, see **fig. 16** for *A. mellifera*). It is very likely that I did not detect several PER-positive cells due to the extremely weak specific staining and the high unspecific background staining of the antibody. Moreover, I could not

unequivocally identify PER glia cells, as I did in the honey bee brains, because of the weak staining in mason bee brains.

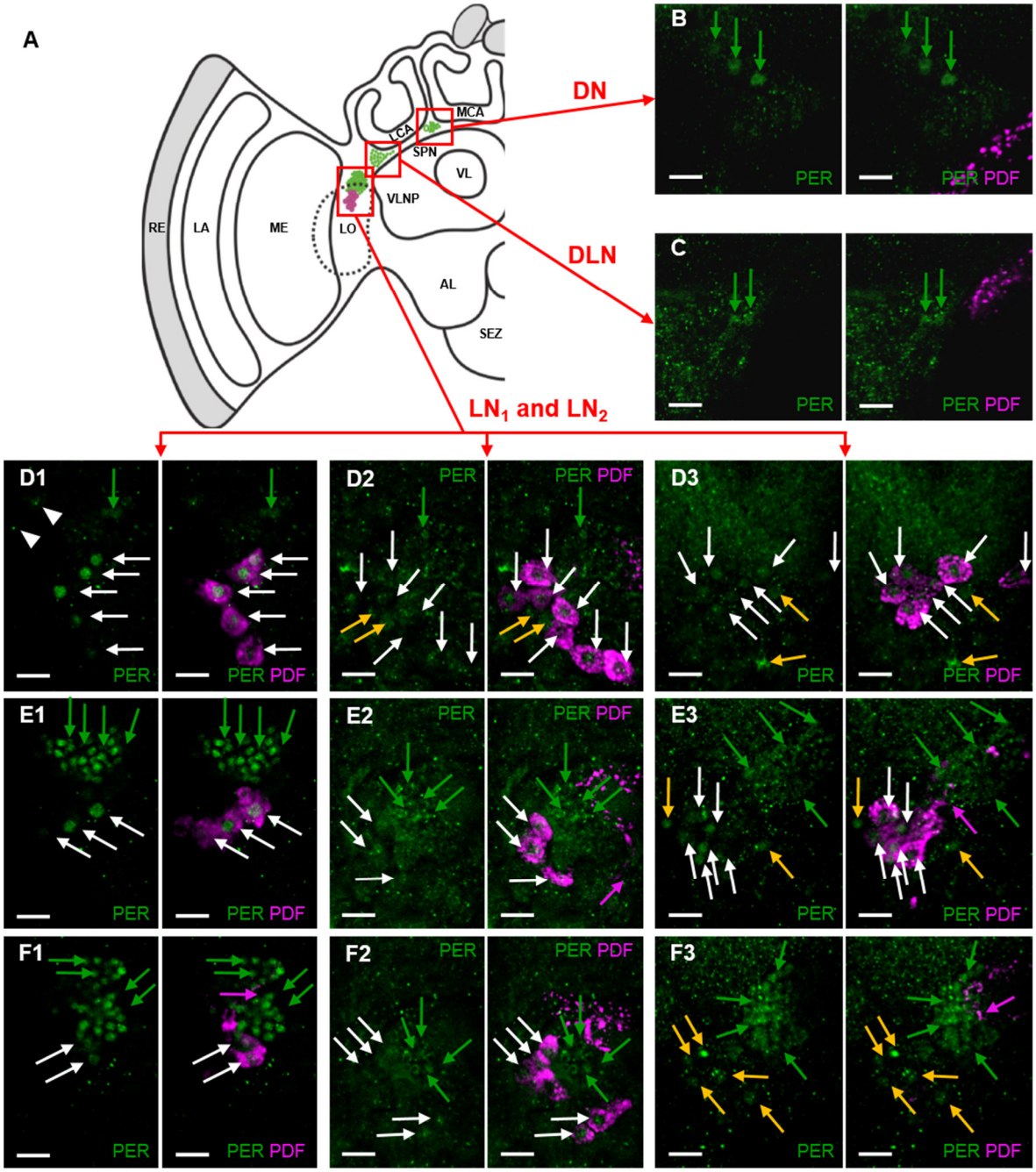

Figure 27: PER neurons in *Osmia*.
PER-positive neurons (green, nuclear staining) in the bee brain. In the LN_2 cluster (white arrows) *per* is co-expressed with *pdf* (magenta = cytoplasmatic staining), while DNs, DLNs, and LN_1s show only PER staining (green arrows). **A:** Scheme of the different clusters in the bee brain. In projection pictures of several confocal stacks, the DN (**B**) and DLN (**C**) cluster in *O. cornuta* are displayed. **D-F:** LN_1 and LN_2 cluster from anterior to posterior. Neurites arising from the LN_2 (magenta arrow) pass closely by the dorsal posterior situated LN_1 cluster (green arrows). Some PER positive stained cells (yellow arrows) appear to be located ventrally of the LN_2 neurons. RE: retina, LA: lamina, ME: medulla, LO: lobula, LCA: lateral calyx of the mushroom bodies, MCA: medial calyx of the mushroom bodies, VL: vertical lobe of the mushroom bodies, SNP: superior neuropils of the protocerebrum, VLNP: ventro lateral neuropils of the protocerebrum, AL: antennal lobe, SEZ: subesophageal zone. Scale bar: 20 µm.

3.4. Development of the circadian clock in bees: PDF network and locomotor activity rhythms in social and solitary bees

The anatomical description of the circadian clock components PDF and PER were very much alike in social and solitary bees, despite the different life style those bees pursue. The different life cycles of the social and solitary bee species is mainly due to differences in development and therefore I thought it likely to find differences in the development of the circadian system, too. Furthermore, I aimed to achieve a better differentiation of the projections of different PDF neurons in early bee development and thereby gain a better understanding of the extremely complex network in the adult bee brain.

PDF staining pattern during pre-adult development

In a comparison of PDF whole mount staining in honey bee brains of different pre-adult developmental stages the PDF neuronal network increased in cell number and complexity of arborizations (**Fig. 28**/**29**). I sampled three larval instars and 5 pupal stages at ZT 0. The bees were raised *in vitro* to improve standardization. In the first larval instar, *pdf* was not yet expressed, but in the third instar, there were already approximately three neurons (2.6 ± 0.4) per hemisphere with somata located at the lateral ventral brain anterior of the LO between the optical lobe (or rather the predecessor structure of the optical lobe) and the central brain (predecessor of the protocerebrum) (**Fig. 28A-C**). For simplicity, I will name larval brain structures like in the adult brain. The arborizations ran into the dorsal brain. In the fifth instar, approximately 5 PDF cells (4.7 ± 0.7) per hemisphere projected further into the lateral dorsal protocerebrum and the dorsal areas of the optic lobes (**Fig. 28D**). In 3-D reconstructions, it was not possible to trace single neurons in the larval brains to either the protocerebral or the optical lobe arborizations, because the PDF neurons project into the dorsal brain in a dense fiber tract (**Fig. 28B**/**C**). This dense fiber tract resembles the ALO (anterio-lobular PDF hub) in the adult bee brain and may be its predecessor. I could show that dorsal arborizations are formed in the larval stage, while the ventral arborizations, the commissures, the LVT and innervations of further structures in the protocerebrum and the optical lobe develop in the pupal stage. In the first pupal stage the main PDF fiber tracts are already structured like seen in the adult brain, with the difference that the cell number is smaller and the fiber tracks are not yet as thick (**Fig. 28E**). Nevertheless the high density PDF network in front of the lobula (ALO) and the LVT tract with its characteristic “loop” are already well developed (**Fig. 28**, white arrows). The arborization network grows and innervations become more dense and complex throughout the pupal development (**Fig. 28 F** and **fig. S4**). In many preparations the fat tissue, which tightly surrounds the pupal brain, could not be removed completely by washing, wherefore there are some unspecific staining signals on the surface of the whole mount (for example at the arrowheads in the projection pictures in **fig. 28**).

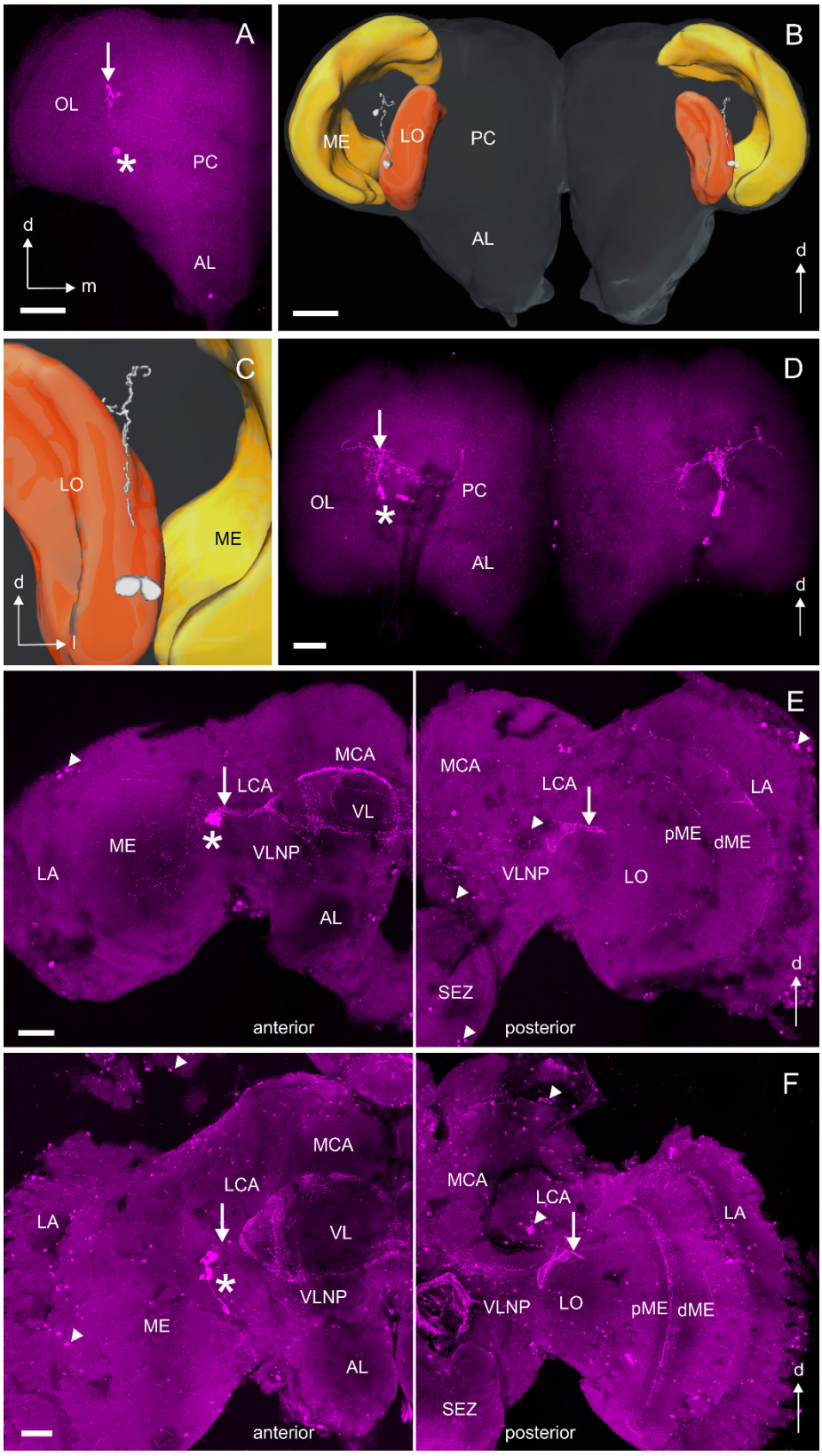

Figure 28: PDF network in honey bees of different developmental stages.
Frontal projection pictures of PDF network (magenta) in whole mount preparations of third instar (**A**) and fifth instar larva (**D**). The 3-D reconstruction (white fibers) of the PDF network in third instar larva shows PDF somata in the ventro lateral brain in front of the LO (lobula) and close to the base of the ME (medulla) (**B** and **C**, which is a detail picture of **B**). The PDF neurons project into the dorso-lateral brain and build a dense network (anterio-lobular PDF hub (ALO) predecessor) from which arborization grow out into the dorsal areas of the protocerebrum (PC) and the optical lobe (OL). In this high density

PDF network the neuronal arborizations intermingle in such a way that no single neuron tracing to either the OL or the PC was possible in the 3-D reconstruction. The PDF network in first pupal instar (**E**) appears already a lot like the network in adult honey bee brain, but number of PDF cells increases and fiber tracts become denser during development until the last pupal stage (**F**). The anterior and posterior half of the whole mount preparations are shown in projection pictures separately. The ALO (white arrow) and the LVT with its characteristic "loop" are already there in first pupal instar. In comparison to larvae brains PDF somata (asterisks) are located more dorsally, which resembles already the organization in the adult honey bee brain. Arrowheads mark unspecific staining due to remains of pupal fat tissue. LCA and MCA: lateral and medial calyx of the mushroom bodies, VL: vertical lobe of the mushroom bodies, VLNP: ventro lateral neuropils, AL: antennal lobe, SEZ: subesophageal zone, LA: lamina, pME and dME: proximal and distal layer of the ME. Orientation arrows: d: dorsal, m: medial, l: lateral

The number of the PDF positive somata (per hemisphere per brain) increased with developmental stage (Kruskal Wallis chi-squared: 67.9, df=7, $p<0.01$; post-hoc test: pairwise comparison using Wilcoxon rank sum test) (**Fig. 29A**). This was also the case in a second trial with a different colony (**Fig. 29B**). PDF neuron number in the later pupal stages does not increase as fast as during early pupal development. In the second trial, the sample size of bees in pupal stages was too low to perform a pairwise post hoc test for all test groups together, wherefore I tested only for differences between larval and pupal stage and for differences among samples of larval instar. Pupae had significantly more PDF cells than larvae (Wilcoxon rank sum test: W=5, $p<0.01$) and cell number increased with age of larval stages (Kruskal Wallis chi-squared: 13.01, df=2, $p<0.01$; post-hoc test: pairwise comparison using Wilcoxon rank sum test). There was no sub-classification of PDF neurons possible by size (see also PhD thesis Esther Kolbe (Kolbe, 2013)) and I followed the approach to define different sub-classes of PDF neurons by identification of clusters arising at different time points in development. For the first trial, I performed a clustering analysis in which I investigated PDF sub-cluster number and how many cells cluster together. This analysis I performed per hemisphere. Number of sub-clusters ($W = 3423.5$, $p<0.01$) and sub-cluster size increased significantly from larval to pupal development ($W = 3207$, $p<0.01$). I found more sub-clusters in brains of older pupae compared to brains of younger pupae (Kruskal-Wallis chi-squared = 18.84, df = 4, $p<0.01$, post-hoc test: pairwise comparison using Wilcoxon rank sum test) and the same for old and young larval instars ($W = 137$, $p<0.01$) (**Fig. 29C** (pupal clusters) and **D** (cluster analysis)). There was no significant difference for the sub-cluster size among different larval and pupal stages respectively, because there are always small sub-clusters throughout pre-adult development of the bee brain. Nevertheless, there seems to be a breaking point in cluster arrangement in P5, when the number of PDF somata increases and the sub-cluster size is reduced (**Fig 29 D** (cluster analysis)). In the following development, the sub-cluster sizes increase again, because the overall number of cells is higher.

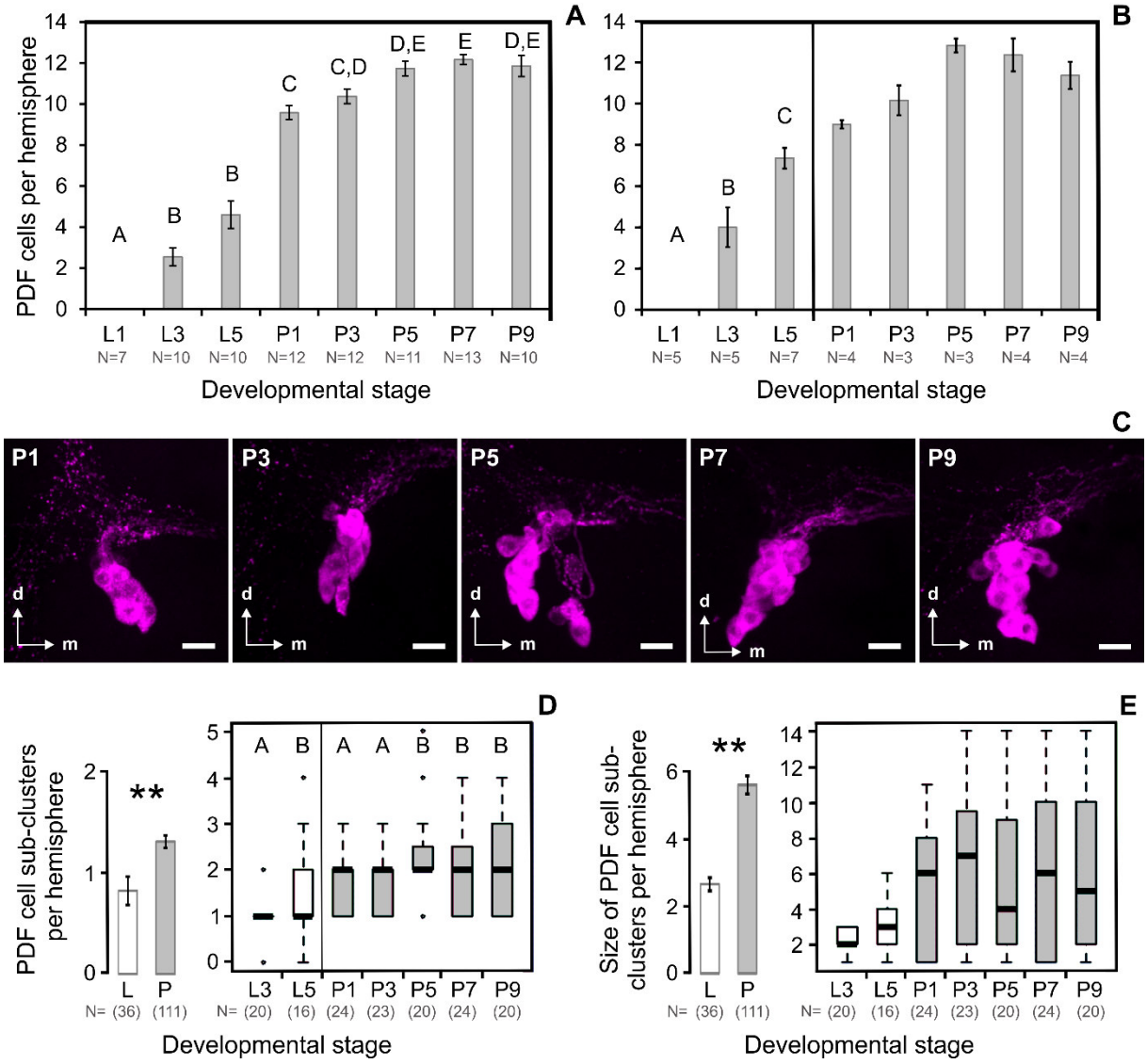

Figure 29: Number of PDF neurons and organization in sub-clusters in different larval and pupal developmental stages.
PDF cell number increases significantly during larval and pupal development (**A** and **B**). In the second trial only larval development could be tested statistically, because of the low sample size of pupal stages. First, third and fifth larval instar (L1, L3, L5) and first, third, fifth, seventh and ninth pupal stage (P1, P3, P5, P7, P9) were tested. Bar plots indicate the number of PDF neurons per hemisphere per brain (N=number of sampled brains). **C:** Examples for differential PDF cell cluster organization in pupal stages (frontal projection pictures of whole mount preparations). PDF cell sub-clusters increase in number and size from larval to pupal development (**D** and **E**, bar plot on the left). Earlier larval and pupal stages respectively display less sub-clusters, while there is no significant difference in sub-cluster size (**D** and **E**, boxplots on the right), but still there are changes in the organization of the sub clusters especially in P5 (N=number of evaluated hemispheres).

Post emergence development of bees of the species *O. bicornis* and *A. mellifera*

In a set of behavior experiments, I assessed if the ontogeny of circadian locomotor rhythms during post emergence development of *O. bicornis* differs from the one in *A. mellifera*. To do so I put cocoons (first trial: 64 cocoons, second trial: 208 cocoons) of *O. bicornis* in the locomotor activity assay tubes (like described in chapter 2.2.2.2.). Environmental conditions in two trials were constant DD, 20 (± 0.5) °C or 19.2 (± 0.1) °C and 60 (±10) % RH. In a third trial (153 cocoons of *O. bicornis*) they were constant DD, 25 (± 0.5) °C and 45 (±10) % RH. In this trial I used monitoring tubes of two different diameters (ø16 and 24 mm), because of equipment restraints. I also monitored 32 newly emerged female worker honey bees taken from a brood comb which was kept overnight in a climate chamber (DD, 20°C, 60% RH, DD) and placed in the monitoring tubes under dim red light. Furthermore I put another experimental group (32 female worker bees) of these newly emerged honey bees in tubes of a monitor positioned inside the experimental climate chamber (constant DD, 30°C, 60% RH) with indirect social contact to a mini hive like described in chapter 3.1. I added this treatment group, because I have observed a very poor survival rate in the experiments before (chapter 3.1.) when the newly emerged honey bees were completely deprived from social contact to their colony. The set up with contact to the mini colony creates a social environment similar to natural conditions and improves survival of young bees (see also chapter 3.1.). The bees (honey bees and solitary bees) have never been exposed to light or oscillating temperature.

I included only bees that showed locomotor activity for at least three consecutive days in the analysis and evaluated actograms by eye, because available statistical tests for periodicity are often not precise enough when only a few days are evaluated (Refinetti et al., 2007). In both cases, with and without the indirect social contact, newly emerged honey bees never displayed circadian rhythms in locomotor activity during the first 2 days after their emergence (**Fig. 30A** and **tab. 7**). Therefore, I pooled the two treatment groups for further analysis. On the other hand, in the three *Osmia* experiments most if not all (100%, 69% and 95% respectively) of the bees showed a rhythm in their behavior during the first 2 days after their emergence (**Fig. 30B** and **tab. 7**).

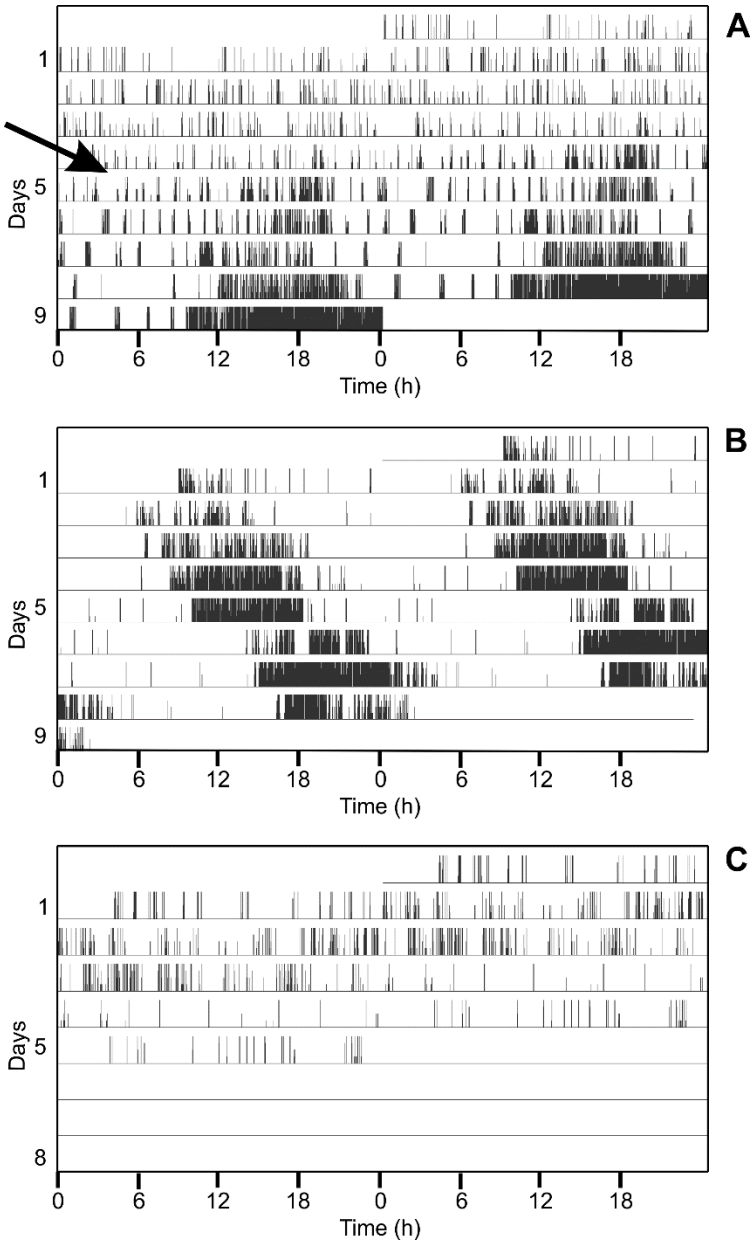

Figure 30: Ontogeny of circadian rhythms in *A. mellifera* und *O. bicornis*.
Representative examples of double plotted actograms for locomotor activity after emergence of *A. mellifera* (**A**), and *O. bicornis* (**B** and **C**), all from female bees. Honeybees never show circadian rhythmicity in their behavior after emergence, but develop a rhythm a few days later (in this example on the fifth day after emergence, arrow). Contrary most solitary bees show circadian rhythmicity right away (**B**) and only a few examples do not develop rhythmicity in their behavior (**C**). These bees often died early.

I again pooled the two treatment groups of the third *Osmia* experiment with two differently sized monitoring tubes, after disproving a significant difference between the treatments ($\chi^2 = 0.47368$, df = 1, p-value > 0.05). In the *Osmia* experiments, females and males were used, because I can only ascertain the sex after the bees emerged from their cocoon. Unlike honey bees, solitary bees differed in their emergence day, wherefore I waited to determine the sex until the experiment was completely finished. As observed in earlier locomotor activity experiments (chapter 3.2.) females tended to display a less pronounced circadian rhythmicity in their behavior. Therefore, I separated the analysis for the

sexes and found that indeed 82% of the small number of individuals, which were arrhythmic during the first two days after emergence, were female. Nevertheless, this seemed to be because more females were evaluated in the three experiments. Only 6.7% (± 3.9) of all measured females in the three trials were arrhythmic compared to 9.2% (± 6.7) arrhythmic individuals among male bees. Moreover, the arrhythmic individuals tended to die early.

Table 7: Rhythms in locomotor activity of newly emerged individuals of social (*A. mellifera*) and solitary (*O. bicornis*) bees.
Environmental conditions, sample size and absolute number as well as percentage of rhythmic individuals of 4 different experiments (1 with *A. mellifera* and 3 with *O. bicornis*). Experiment *Apis* and *Osmia3* had two different treatment groups, which were pooled together after disproving statistical differences between treatments. Analysis is also displayed for males and females separately.

	Apis	***Osmia* 1**	***Osmia* 2**	***Osmia* 3**
Environmental conditions	30°C, 60 % RH	20°C, 60 % RH	19.2°C, 60 % RH	25°C, 45 % RH
N	40	17	50	20
Rhythmic individuals	0	17	34	19
Percent of rhythmic individuals	0%	100%	68%	95%
N (male)		8	10	13
Rhythmic males		8	8	12
Percent of rhythmic males		100%	80%	92%
N (female)	40	9	40	7
Rhythmic females	0	9	26	7
Percent of rhythmic females	0%	100%	65%	100%

4. Discussion

4.1. Locomotor activity rhythms of honey bees in social context (see also Beer et al., 2016)

In this study, I introduced a new set up for monitoring individual locomotor activity of honey bees in context with the colony rhythm. An IR (infrared)-beam based activity acquisition system was combined with a single frame colony, in which I monitored temperature rhythms. Thereby I could show that my set up mimics the natural conditions in many aspects and I could observe not only social synchronization of the individuals to the colony, but also that social cues seem to be more important than light, when the bees are exposed to competing Zeitgebers. In the trials with newly emerged honey bees I showed that the set up can compensate social separation stress, which causes increased mortality of young bees. Further, the ontogeny of circadian rhythms seemed to be accelerated in young bees with social contact, as they displayed diurnal activity rhythms significantly earlier.

Mini-colony mimics a natural colony in many aspects

While I had to neglect free flying conditions for foragers due to the limited space capacities in the laboratory, I could observe many aspects of a naturally functioning colony in the mini-colony. The mini-colony is a reduced version of a natural colony with up to 1000 bees in which the queen was substituted with a queen pheromone, which means that the colony is not fully functional. But former studies demonstrated that the absence of a queen had no effect on thermoregulation in a beehive (Kronenberg and Heller, 1982). Therefore, the extent to which the mini colony mimics a natural bee colony had to be tested. The following observations in our set up were in favor of a normal colony function: (1) bees in the mini hive raised the brood normally, so that the next generation is still alive and apparently healthy after 3 weeks in the box. (2) Bees showed normal dynamics in temperature regulation inside the mini hive. Like in a natural colony in which honey bees typically keep their brood at constant high temperatures of approximately 32–36 °C, but put less effort in heating the food cells (Kleinhenz, 2003; Kronenberg and Heller, 1982; Simpson, 1961; Stabentheiner et al., 2010), I observed different temperature amplitudes at the brood and at the food storage cells. Forager and hive bees that have duties other than handling the brood are rhythmic and usually stay outside the brood center (Bloch et al., 2001; Stabentheiner et al., 2010), coinciding with the sites where I observed the highest temperature oscillations (at the food sensor). The bees in my set-up could not forage outside of the mini hive box. Therefore, foragers produced daily oscillation patterns in temperature because their body temperature raises during their activity phase, as it has been observed in studies on isolated forager bees (Fuchikawa and Shimizu, 2007b). The recorded brood temperature lay between 31.1 and 34.8 °C, which indicated that few nursing workers could actively regulate the brood temperature, which was important for a normal development (Groh et al., 2004; Jones et al., 2005; Tautz et al., 2003). The slight temperature drop detected by the brood sensor was probably due to the small number of bees in the mini hive. Other studies of hive temperature in single frame colonies

yielded similar results (Kronenberg and Heller, 1982). In summary, my mini colony appears suitable to create a simulated colony environment for the singly housed bees. This was confirmed by the higher survival rates of newly emerged honey bees with social contact as compared to those in isolation.

The set up facilitates high data throughput

Classical observations (Beier and Lindauer, 1970; Crailsheim et al., 1996; Kefuss and Nye, 1970) are inexpensive but time-consuming and therefore not feasible in case of large data sets. Activity data sets can be evaluated more efficiently with automated systems. Such systems use for example video tracking software (Eban-Rothschild and Bloch, 2015; Gilestro, 2012; Sauer et al., 2003), but there are some disadvantages of these monitoring systems like limited capacity of individual tracking, high purchase costs and susceptibility to errors, if videotaping conditions are suboptimal. I therefore decided for an automated system based on movement detection by IR-barrier beams, because of its cost-efficiency and simplicity in application. The DAM system by TriKinetics has been successfully used for decades and facilitated high data throughput studies of locomotor activity to gather consolidated findings in the field of chronobiology (Schlichting and Helfrich-Förster, 2015). My set up combines a “natural like” simulation of social contact in the bee colony with the DAM System individual activity data acquisition.

Impact on non chronobiological studies in young bees

The impact of social contact on the behavior of bees is not only interesting for chronobiological studies. Newly emerged bees are very sensitive to stress and have a high mortality rate when they are caged in the laboratory (Milne Jr, 1982). In my experiments, I saw a clear effect of separation stress on newly emerged individuals (day 1), which could be compensated providing indirect social contact. It has been shown that nurses promote the development of young bees probably by feeding jelly (Naiem et al., 1999). But I could show that the high mortality among the socially isolated newly emerged bees in my set up is not related to malnutrition because all test subjects were provided with the same food and prevented from conducting trophallaxis with bees in the mini colony by a double mesh separation system. During the very early stage of adulthood the separation from a social environment seems critical for survival and this is not only due to nutritional reasons. In this respect, my set up can help to realize experiments with newly emerged bees as test subjects.

Contact with the mini colony enables social synchronization

Most interestingly, the individually housed bees can socially synchronize their activity rhythms with the mini colony rhythm. This was evident under DD conditions, in which the activity rhythms of forager-aged bees free-run in parallel to the temperature rhythms of the colony. In an experiment in which I exposed individual bees to a LD cycle phase shifted to the colony rhythm I investigated whether social cues are stronger Zeitgebers than external LD cycles. Another control group was exposed only to the

LD cycle, but had no contact to the mini colony. I found significant differences in the rhythmic behavior of these two groups, independently of their age (even though the responses of forager-aged and nurse-aged bees were quite different with respect to rhythmicity). Forager bees in isolation displayed exactly the same percentage of rhythmicity (84 %) as shown in an earlier study (Ludin et al., 2012), whereas the indirect social contact in my set up raised the number of rhythmic bees in both age groups. Forager-aged bees with social contact displayed an unusually low response to the light signal but instead phase delayed their activity rhythm in synchrony with the hive rhythm. This clearly showed that the social Zeitgeber is stronger than the LD cycle. The overall lower activity of bees with social contact may be a response to the conflicting Zeitgebers, which gave two different entraining inputs. Nurse-aged bees without social contact did not develop an immediate activity rhythm under LD cycles. They needed about 7 days to synchronize with it. The small and delayed response to light may be explained by the following reasons: (1) their visual system is not fully matured, (2) the connections between the visual system and the circadian clock are not yet established or responsiveness to light is differently modulated in the nurse-aged bees and (3) nurse bees lack a fully operating clock right after emergence. The first point is unlikely, because the electroretinogram of nurse-aged bees appears normal (Ben-Shahar et al., 2003). But Ben-Shahar et al. also showed that nurse-aged bees do not display a forager-like photo tactic response, which speaks for reason (2). If so, the earlier diurnal activity patterns in nurse-aged bees with social contact may also be due to the fact that the light input cannot entrain very young bees, but social cues can. My observation that bees need about 4 days to establish a rhythm supports also the third option, a lack of an operating clock right after emergence. This is in line with previous studies (Bloch et al., 2001; Eban-Rothschild et al., 2012). Eban-Rothschild et al. (2012) conclude in their study that exposure to the colony environment during the first 48 h after emergence is mandatory for a proper development of the circadian clock in honey bees. They showed this in a comparison of two test groups: one in social contact, the other in isolation for the first 48 h of their adult life. When isolated in the laboratory afterwards only bees, that had social contact before, displayed circadian rhythms in their behavior right away. In line with this study I also observed that nurse-aged bees with social contact show diurnal activity patterns significantly earlier, namely from the second day of their adult life on. Nevertheless, I cannot rule out that the connections between the visual system and the circadian clock could additionally be poorly established in young bees, or the clock network is simply not yet synchronized.

Half of the nurse-aged bees in contact with the colony started to be very active when temperature dropped in the mini hive, which seems as if these bees tried to counteract the drop in hive temperature. Indeed, nurses maintain temperature at the brood constant by either producing heat via clustering and indirect flight muscle contractions if the temperature is too low or by fanning if the temperature is too high (Esch and Bastian, 1968; Simpson, 1961; Stabentheiner et al., 2010). The bees in my study were hindered to cluster in the monitoring tubes and therefore may have attempted to produce heat via raising their locomotor activity. The fact that bees in my set up initiated this behavior on the second day is in line with Stabentheiner et al. (2010), who showed that bees are only able to actively thermoregulate from day 2 onwards. Earlier studies showed that the temperature thresholds for engaging in thermoregulation are variable in colonies with diverse genetic background,

a fact that increases the efficiency of controlling temperature in the hive (Jones et al., 2004; Myerscough and Oldroyd, 2004). Since the colony I used for this experiment also had a diverse genetic background by natural mating, it is likely that only some bees started heating behavior when temperatures dropped below 29 °C, while others may have started later.

It was surprising that both forager- and nurse-aged bees in social contact barely responded to the LD cycle, because light is a strong Zeitgeber most organisms can be entrained to (Aschoff and Pohl, 1978). One might expect that especially nurses, which are used to a dark surrounding in the hive, would be particularly sensitive to light pulses. So far, only one study in honey bees indicated that social cues have more importance than light (Fuchikawa et al., 2016). In this study, a field foraging colony was exposed to conflicting LD and social rhythms (foraging activity). Monitored under constant conditions in the laboratory afterwards, foragers and nurses showed the same entrainment pattern to the foraging cycle. On the other hand, a different group of caged nurse-aged bees that only experienced the LD cycles showed entrainment to the LD cycles. Fuchikawa et al. concluded that social cues override the light input in young nest bees. In addition, my study showed that social cues can be stronger Zeitgebers than light also for forager-aged honey bees as long as they are prevented from foraging outdoors.

Putative factors responsible for social synchronization in the set up

As I mentioned in the introduction the necessary cues for social synchronization in a beehive are not yet elucidated. There are indications that contact over microclimate (temperature, CO_2, relative humidity) or vibration (Moritz and Kryger, 1994; Simoni et al., 2014) as well as pheromones, which are used in nest mate communication in a variety of situations, e.g., swarming or alerting behavior (Free, 1987) could play a role in the social synchronization of activity rhythms. Olfactory cues have been shown to be involved in social entrainment in rodents (Goel and Lee, 1997) and, to some extent, in fruit flies (Krupp et al., 2008; Levine et al., 2002). My experiments help to narrow down the list of potential factors responsible for social synchronization. For example, I can exclude physical contact as factor necessary for synchronization, because the bees were physically separated by the double mesh system and nevertheless synchronized with each other. Other studies support this conclusion (Fuchikawa et al., 2016; Moritz and Kryger, 1994). Temperature cycles can be ruled out as sole synchronizing cue in my set up as well, because I measured only temperature amplitudes of ~2 °C in the glass tubes. These fluctuations are well below the tested minimum (6–10 °C) for complete entrainment of the circadian clock of honey bees (Fuchikawa and Shimizu, 2007a; Moore and Rankin, 1993). My newly developed bioassay can be used in future studies to unravel the exact mechanism of social entrainment in honey bees, by manipulating different putative cues for social entrainment.

Outlook on future applications of the set up

My set up can be modified in several ways for further study social effects on the locomotor activity rhythms and entrainment of circadian rhythms of individual bees. By removing the net on the tube cap

that provides the double mesh system between the tubes and the mini colony, one can monitor the effect of direct tactile contact and trophallaxis on behavior. CO_2 or pheromones could be prevented from entering the tube by closing the side facing the mini hive with a volatile-tight membrane, or visual signals can be blocked by a shield introduced between individual bees and the colony bees. Furthermore vibrational signals can be eliminated by removing the wooden clamp that connects the monitor to the hive box and introducing a material that buffers vibrations. Monitoring bees in indirect contact is of great advantage over monitoring in groups with tracking systems: one can easily perform manipulation studies without affecting the hive bees. This permits studies like different feeding assays in social context with the mini colony, where all bees in the monitors can have their individual food provisioning and the double mesh system prevents them from carrying out trophallaxis. Moreover, my set-up makes bee sampling for molecular analysis easier, as it saves the experimenter from having to open a beehive. This would be especially interesting for analysis of clock gene expression in differently aged honey bees to gain a better understanding of how the clock input works in different life stages.

In summary, I developed a new and easily applicable set up for investigating individual locomotor activity rhythms in social context under controlled laboratory conditions. Although my set-up does not allow the bees to perform age related tasks and thus does not fully simulate the natural situation in a honey bee colony, it is well suited to study particular effects of the social environment. For example, it can be used to identify the cues needed for synchronization of singly housed bees with the rhythms of the colony. Furthermore, my system allows manipulations to the bee's social system that can be performed even in a laboratory with space limitations. Thus, my set up could have a great impact on the realization of laboratory experiments aimed at understanding honey bee behavior and the influence of social interactions in a honey bee community.

4.2. Emergence rhythms and locomotor activity rhythms of solitary bees

In my studies, I showed for the first time that emergence of *O. bicornis*, an adult overwintering bee species, is governed by the circadian clock and that it is entrainable by temperature cycles. This has been shown so far only in bees overwintering in pre-pupal stage (Tweedy and Stephen, 1970). I further report the first locomotor activity rhythms in solitary bees, which are entrainable by LD cycles. In cooperation with Mariela Schenk (Neurobiology and Genetics, University of Würzburg) I discovered that temperature has a strong synchronizing effect on emergence rhythms, while light has none, or close to none, and emergence rhythms under LD conditions remind rather of a free running rhythm without any synchronizing environmental factors. The strong dependency on temperature as trigger for synchronized emergence rhythms might be a conserved property of emergence behavior in solitary bee species and other insects. This may be crucial information for conservation management and pollination services.

Locomotion and emergence activity regulated differently

Locomotor activity of adult solitary bees can be synchronized by LD cycles as I have shown for *O. bicornis*. Light is an important environmental time cue, which can synchronize activity rhythms of different vertebrates, plants and insects, including honey bees, with their environment (Aschoff and Pohl, 1978; Frisch and Koeniger, 1994; Ludin et al., 2012; Moore and Rankin, 1993). Nevertheless light has not been shown to trigger daily rhythmicity in emergence of the solitary bee *M. rotundata*, a bee species with pre-pupal winter diapause (Tweedy and Stephen, 1970), and the same was the case in our experiment with *O. bicornis* (see supplementary **fig. S1/2** of joint study). In line with our observations Tweedy&Stephen (Tweedy and Stephen, 1971) report that a rhythm in oxygen consumption in different developmental stages of a solitary bee (*M. rotundata*) was missing under LD cycles. In later experiments, they admit a slightly synchronized reaction to light after keeping the bees for a very long time under LD cycles, but doubt the biological relevance, because of the high inter-individual variance in FRPs and the failure to phase reset oxygen consumption rhythms by light signals. In comparison, in various insects, like *Drosophila*, European corn borer, cockroach and honey bee, oxygen consumption has been shown to be synchronized by LD cycles (Beck, 1963; Rensing, 1966; Richards and Halberg, 1964; Southwick and Moritz, 1987).

In *Drosophila melanogaster* it was shown that light cycles can entrain emergence rhythms (Brett, 1955). Lankinen (Lankinen, 1986) showed that emergence rhythms in *Drosophila littoralis* were entrained by the switch from DD to LL conditions. They observed a circadian rhythm in emergence damping in 2-7 days, which is very similar to my results with a damping in 5-7 days after the switch from temperature cycles to constant conditions. In Mariela and my joint experiment we could keep the temperature completely constant (± 0.01 °C) and therefore can largely exclude that the entrainment reaction in solitary bees was due to a small temperature pulse created by the lights in the incubator in light-dark cycles. Indeed we could show that the circadian system is extremely temperature sensitive,

because we saw a free-running emergence rhythm lasting several days triggered by the last temperature raising step (ΔT=1°C) before emergence in LD (**Fig. S1/2**). This sensitivity is even higher than reported for *M. rotundata* (ΔT=2°C) (Yocum et al., 2016). Nevertheless in our experiment we cannot completely exclude a minimal synchronizing effect of light on the emergence rhythms, when the bees would be kept longer under LD cycles, like it was demonstrated for oxygen consumption rhythms in *M. rotundata* (Tweedy and Stephen, 1971), but light has for sure evanescent importance in synchronizing emergence in *O. bicornis* compared to temperature. Anyway, the biological relevance of light signals, which can hardly penetrate the cocoon (measurements performed by Mariela Schenk, personal communication) and therefore will not reach through the tightly sealed nests in wood cracks, is doubtful. Entrainment of locomotor activity rhythms in honey bees and possibly other bee species needs a minimal temperature amplitude of 7-10°C (Fuchikawa and Shimizu, 2007a; Moore and Rankin, 1993). Properties of entrainment of locomotor activity stand in contrast to the high temperature sensitivity and low light sensitivity in emergence rhythms in the solitary bees (our results and (Tweedy and Stephen, 1970)). This demonstrates that the circadian clock governs both behaviors, but different regulatory mechanisms may be present.

High temperature sensitivity and low light sensitivity in emergence reaction conserved among various insect species

The observed high temperature and low light sensitivity in emergence rhythms seems to be conserved among different solitary bee species, because the same was found for pre-pupal diapausing bees (Tweedy and Stephen, 1970). This might not only be a shared trait among bees, but rather common among various insects. Data of emergence rhythms in *D.* melanogaster raised under natural conditions were used to model importance of different environmental factors for emergence. This model, which included the factors temperature, humidity and light, evidenced that temperature synchronizes emergence rhythms of *D. melanogaster* stronger than light (Ruf, 2016). Unfortunately there is no report of emergence rhythms in honey or bumble bees. Conclusions on an arrhythmic emergence behavior have been drawn by a lack of rhythm in mated queen behavior in the colony and oviposition (Eban-Rothschild et al., 2011; Free et al., 1992; Harano et al., 2007; Johnson et al., 2010) and preliminary studies in honey bee colonies support this assumption (personal communication Guy Bloch). On the other hand daily oviposition rhythms in colonies of eusocial stingless bees were reported, but it is not clear if this rhythm depends on the brood cell construction rhythm or if it is independent from this activity (Oda et al., 2007). Social bees have no obligatory diapause but rather yearly rhythms of swarming and winter dormancy or in case of bumble bees only the queen undergo winter diapause (which can be omitted under certain laboratory conditions) (Beekman et al., 1999; Seeley, 2010). They raise their brood under constant high temperature conditions in the colony, which makes the need for a strong temperature response and synchronized circadian emergence behavior per se evolutionary nonessential and rhythmicity in emergence may have been lost in social bees.

Sex differences in rhythms of *O. bicornis*

In my experiments male *O. bicornis* emerged 4 days earlier than females, which is consistent with studies under natural conditions (Bosch and Blas, 1994; James and Pitts-Singer, 2008). Females had a slightly faster free-running emergence rhythm and the rhythm power in female emergence was lower than in males, which might simply be due to females emerging a few days later and the last time signal they experienced lies further back. In the preliminary locomotor activity records of individual bees, females tended to have a longer period and entrainability to LD cycles seemed less effective in females. In studies conducted with *D. melanogaster* males showed an earlier onset of morning activity and also a shorter FRP (free-running period) (Helfrich-Förster, 2000). Similar to my findings in bees, hamster males show greater ability to entrain to LD (Davis et al., 1983). Hamster females like human women prefer to start their activity in the morning statistically before males/men (Davis et al., 1983; Duffy et al., 2011), which stands in contrast to findings in *Drosophila* (Helfrich-Förster, 2000). Moreover women tend to display a shorter FRP in cycling production of the sleep hormone and body temperature cycles (Duffy et al., 2011). It would be interesting to test whether sex differences in the circadian system in bees rather share properties with other insects or with mammals, as the bee clock has been shown to have some vertebrate typical attributes in the molecular clock and the reaction to light shifts (Aschoff and Pohl, 1978; Cheeseman et al., 2012; Ludin et al., 2012; Rubin et al., 2006; Weinstock et al., 2006). Further studies are needed, because of the low sample size presented here, which is one for the population emergence rhythm behavior and 18 in the individual locomotor activity experiments. This is not sufficient to draw definite conclusions.

I observed a tendency that males emerged shortly before females during 24 h periods (supplementary **fig.S5**/**A**) under DD conditions with previous temperature entrainment. This sex specific circadian emergence rhythm was reminiscent of the difference between male and female annual emergence. Since there are several indices that circadian and seasonal timing in insects might share (at least partially) the same clock mechanisms (reviewed in Danks 2005, Kostal 2011) and clock genes have been proposed to be the basis of annual time keeping in mammal calendar cells (agranular secretory cells of the pars tuberalis of the pituitary gland) (Lincoln et al 2003), I tested if circadian and annual emergence in bees would share this sex specificity. This might point to a shared time keeping mechanism in circannual and circadian clock. Nevertheless, a control experiment I conducted with bees emerging under temperature cycles at lower temperatures, which should maximize the daily gate of efficient emergence time, showed no significant results (supplementary **fig.S5**/**B**). The temperature cycles I chose were the same as the last temperature cycle in the entrainment of the emergence experiment under DD. The analysis of emergence phases in the mean day of the control experiment with temperature cycles did not support a potential sex difference. This means that annual emergence rhythms and circadian emergence rhythm do not share this property of males emerging earlier and the difference I observed under DD was either due to the small number of bees emerging per day or the desynchronization of individual rhythms without a Zeitgeber. In solitary bees, there seems to be no biological reason for males emerging a few hours before the female, like it has been observed in some *Clunio* species (Oliver, 1971). In these species the males emerge shortly before the females and help these out of their pupal skin. *Osmia* females do not need assistance during emergence, and the males

emerge in most cases even days earlier and wait for the females at the nest site, to ensure their reproductive success (Bosch and Blas, 1994; James and Pitts-Singer, 2008).

Daily temperature rhythms in ecological and economic applications

My studies on the circadian clock of solitary bees may contribute to improving conservation and pollination management. With 16,000 different species (Michener, 2000) bees are among the primary pollinators for the majority of flowering plants (Ollerton et al., 2011) and are therefore of particular value for ecological systems as well as agriculture (Biesmeijer et al., 2006; Klatt et al., 2013; Potts et al., 2010). Lately attention grows towards the commercial value of wild solitary bees (Garibaldi et al., 2013, 2014), but intensified farming praxis has turned out to reduce their abundance and alongside crop yield (Garibaldi et al., 2011; Holzschuh et al., 2006, 2016). Therefore, it is important to understand different aspects of their natural behavior and develop adequate bee breeding protocols. For hibernation it is common practice to store solitary bees (as single cocoons or inside entire nests) indoor at constant temperatures around 4-6 °C, but studies have shown that survival of solitary bees in long term storage can be improved by exposing them to fluctuating temperature regimes with a daily pattern (Rinehart et al., 2013; Yocum et al., 2012). Moreover development is accelerated in daily fluctuating temperatures (Radmacher and Strohm, 2011). This demonstrates the importance of considering the circadian clock of bees when establishing protocols for pollination management. With our study, we can affirm that temperature is a main environmental trigger for synchronized emergence also in adult diapausing bee *O. bicornis* and raising protocols should include temperature cycles during storage to achieve a natural like emergence reaction and thereby improve bee fitness.

In summary, I could show that daily emergence in *O. bicornis* is governed by the circadian clock and entrainable by temperature rhythms. Basic properties of circadian rhythms in locomotor activity and emergence are consistent with findings in other insects. Especially the high sensitivity of the clock to temperature cycle of low amplitude in synchronizing emergence rhythms may be a conserved trait among different insects. This knowledge may help to improve wild solitary pollinator management. My findings on sex specific differences are still preliminary, but the established methods of locomotor activity and emergence monitoring provide the basis for continuative studies.

4.3. PER localization pattern in bees (see also Fuchikawa et al., 2017)

My studies on the PER localization pattern in bees have contributed significantly to several points in the neuronal characterization of the circadian network. First, they provide, together with the PDF staining pattern, the best description of the circadian system in the bee brain that is now available. Thereby the stage is set for further investigations of the integration of the circadian clock in functions like time memory, the time compensated sun-compass navigation and dance communication. Second, although a detailed analysis of the PER localization pattern in solitary bee brains was not successful I achieved to describe a general staining pattern that seems similar to the one found in honey bees. Third, finding similarities of the PER staining pattern in bees and different insects suggests a common ground plan for the circadian system conserved among insects. Fourth, similar to the mammalian system, in the honey bee we could not find evidence of gated entry of *am*PER into the nucleus, which might point to an adaptive function of having different clock genes in different systems. Fifth, testing the antibody specificity in different insects demonstrates the potential but also the limits of this antibody validation approach, because the recognition of PER with the anti-*am*PER antibody in *Drosophila* seems better than in *Osmia*, which are closer related to *A. mellifera*.

Cross species recognition studies for antibody specificity validation

The validation of the anti-*am*PER antibody was conducted via four different methods: Western Blots (WB), Immuno Precipitation (IP) with anti-*am*Per and anti-*am*Cry antibodies (see for these methods and results Fuchikawa et al., 2017), immunocytochemistry on wild-type and per^{01} mutant *Drosophila* flies and testing the antibody on honey bee brains after pre-absorbing it on *Drosophila* per^{01} mutant embryos. Staining intensity (in immunofluorescence and WB) was strong during the late night and almost gone in the middle of the day, demonstrating daily cycling and an involvement of the stained protein in the circadian clock. IP and sequencing of Western Blot extracts hardened the case that the used antibody actually stained PER. The last part of the validation, namely identifying the well characterized staining pattern of PER in *Drosphila* wildtype flies and the lack of staining with the anti-*am*PER antibody in period mutant flies, gave additional proof.

My immunostainings confirm and extend earlier studies in honey bees which used antibodies raised against PER from other insect species, like the full-length *D. melanogaster* PER protein or a synthetic 14-mer peptide corresponding to a fragment of the most conserved region of the Chinese tussar silkmoth *Antheraea pernyi* PER (Bloch et al., 2003; Závodská et al., 2003). *Am*PER has 55% amino acid sequence similarity to *dm*PER (Toma et al., 2000) and differs in only one residue from the corresponding 14-mer peptide of *A. pernyi* making it very likely that these antisera recognize *am*PER, at least in some configurations. My successful staining experiments on the well-characterized PER-expressing neurons in *Drosophila* brains incubated with the new anti-*am*PER antibody further confirms the similarity of bee and fly PER. Bloch et al. report already 2003 extensive nuclear staining in the optic lobes and the central brain using anti-*dm*PER antibody (Bloch et al., 2003). These cells I could now identify as to be of non-neuronal possibly glial nature. In contrast, I could only in a few cases confirm the finding of cytoplasmic PER staining in the dorsal brain like it was observed before by Bloch

and co-authors (Bloch et al., 2003) and Závodská and co-authors (Závodská et al., 2003) using anti-*ap*PER antibodies. The staining pattern of cytoplasmic period expression in the dorsal brain is typical for some lepidoptera (Sauman and Reppert, 1996; Sehadová et al., 2004). Nevertheless, one cannot be sure that species-specific antibodies always recognize the homologue protein in another species. Indeed, cross-species antibodies may provide weaker epitope recognition, could fail to immunostain some PER-expressing cells and increase the risk of false positives. For example, it has recently been shown that antisera generated against part of the *D. melanogaster* PER successfully immunostained clock neurons in related *Drosophila* species such as *D. simulans* and *D. yakuba*, but not in the less taxonomically related *D. ananassae*, *D. triauraria*, *D. pseudoobscura*, *D. willistoni*, *D. virilis*, *D. littoralis* and *D. ezoana* (Hermann et al., 2013). Anti-PER and anti-TIM antibodies stained apart from clock neurons also the cytoplasma of neurosecretory cells in the PI (pars intercerebralis) and PL (pars lateralis) in *Drosophila* (personal communication Charlotte Helfrich-Förster), which reminds of the few cells in the bee with cytoplasmic PER staining in the PL. Curiously the PER-positive neuronal clusters in solitary bee brains were stained rather weakly with a low signal to noise ratio, although one would expect that the PER proteins in honey bees and mason bees would be more similar as the species are closer related to each other than to *Drosophila* (Hedtke et al., 2013). Due to the low signal to noise ratio in the PER staining in solitary bees I cannot be sure, if the number of stained cells are representative or if I have missed some cells. Nevertheless, as the number of PDF-positive cells are the same for foraging *Osmia* and *A. mellifera* it is likely that the number of PER-positive cells is also similar in solitary and social bees. Another problem is that I could not unequivocally distinguish between neurons and glia cells. A precise analysis for cell number and –type as well as daily cycling in expression is only promising after improvement of the PER staining protocol in solitary bees. By application of antibody, pre-absorbed on embryos of Drosophila per^{01} mutant flies, I was able to reduce unspecific background staining. Unfortunately, the staining intensity of specific signals was reduced alongside. Using higher concentrated antibody solutions (1:100, not shown in the results) I did not gain a significantly better signal to noise ratio. In case of PER staining in *Osmia* the approach using whole mounts might not be successful for characterizing details of the expression pattern. I suggest preparing vibratome sections of solitary bee brains in order to improve the background to signal ratio to a satisfactory level. Another problem of cross-species studies is that the right protein homologue may be stained but the immunofluorescence may be rather weak in some cells with less protein due to lower antibody specificity. The lower specificity of the anti-*Drosophila* and anti-*A. pernyi* PER antibodies may have also compromised detection in cells showing weak nuclear *am*PER labelling such as the LN_2 cells that co-express PDF in previous studies in *A. mellifera* (Bloch et al., 2003; Závodská et al., 2003).

*Am*PER staining pattern reveals common neuroanatomical principles in the insect circadian clock

The staining pattern for PER in bees consists of two clusters in the lateral brain adjacent to the medulla and two clusters more dorsally located closer to the calyces of the mushroom bodies. In cells of one lateral cluster PDF is co-expressed with PER. Furthermore, many additional PER-positive

signals in non-neuronal, putative glia, cells were found throughout the cortex of the brain, optic lobes and antennal lobes and PER-positive cells were found in photoreceptor cells. This general pattern in immunofluorescent staining is consistent with different insect species (e.g. *D. melanogaster*, *Pachymorpha sexgutta, Rhodnius prolixus*). A similar pattern of PER expressing cells exists in *D. melanogaster* (Helfrich-Förster, 2005b; Helfrich-Förster et al., 2007b; Siwicki et al., 1988). The lateral central brain of *Drosophila* includes three distinct neuronal clusters, the ventrally located small and large LN_v and the more dorsally LN_d. Additionally, three clusters of PER neurons are located in the dorsal brain, named dorsal neurons DN_1, DN_2 and DN_3. The ventrally located small and large LN_v of *Drosophila* co-express the neuropeptide PDF, except for one small LN_v (5^{th} s-LN_v) that is PDF negative. Similarly, the ventral group of lateral neurons in *A. mellifera* that we named LN_2 co-express PDF and are most probably homologous to the LN_v of *D. melanogaster*. A remarkable difference is that the honey bee clock network contains significantly more cells. The number of PER/PDF co-expressing neurons is eight per hemisphere in *D. melanogaster* and about twice this number in the honey bee. The difference is even larger for the PDF-negative PER-positive lateral neurons: *D. melanogaster* possesses six LN_d neurons whereas the LN_1 cluster in the honey bee consists of 105–120 neurons. Due to this large difference in number, one cannot be certain whether the fly LN_d and bee LN_1 are homologous in nature. The more dorsally located DLN of *A. mellifera* includes approximately 70 cells and might be homologous to the approximately 40 DN_3 cells of *D. melanogaster*, because they are rather wide spread throughout the dorsolateral brain in comparison to the two lateral cell clusters and extend into more posterior parts of the brain. Thus, they are sitting on top of the lateral protocerebrum very similar to the DN_3 of *D. melanogaster*. Nevertheless, the bee DLN cells seem to be located less dorsally than the fly DN_3. This might be due to the much larger bee calyces occupying more space in the dorsal brain, which causes the DLN cells to appear lower and more lateral than in *Drosophila*. Finally, the approximately 15 bee DN cells correspond most probably to the fly DN_1. These cells are not only similar in number, but are also located in the same position anterior of the calyces and dorsal of the PDF fibers. It was not possible to unequivocally identify neurons in the bee dorsal brain that correspond to the two fly DN_2, which virtually sit on the PDF fibers. This does not necessarily mean they do not exist. It could as well be that they are closer to the DN cells and these cell clusters merged. In some cases in the honey bee and many cases in solitary bees I identified PER-positive/ PDF-negative cells located separately from the LN_1 cluster and ventrally of the LN_2 cluster. These cells might be homologues to the 5^{th} s-LN_v in *Drosophila*. Co-staining with another neuropeptide, ITP (Ion Transport Peptide), which has been shown to be expressed only in PDF-negative LN_v in Drosophila and one cell of the LN_d cluster (Hermann et al., 2013), might confirm this assumption. Further characterization of the PER-positive neurons in the bee brain with other neuropeptides might reveal a more defined differentiation between the clock clusters.

In the beetle *Pachymorpha sexgutta* nuclear PER immunostaining in two lateral neuron clusters close to the medulla and many putative glia cells in the optic lobes were described (Frisch et al., 1996). Furthermore, one neuron cluster in the lamina and four cell clusters with cytoplasmic PER staining, which were suspected to be mostly neurosecretory cells, were identified in the dorsal brain of the beetle. The PER-positive lateral neurons were located in close vicinity to PDF neurons, but no co-

localization of PER and PDF was seen. It is likely that PER levels were below detection level in this case like already assumed for the earlier honey bee studies (Bloch et al., 2003), because the same antibody, raised against whole length *D. melanogaster* PER, was used.

In the brain of the hawkmoth, *Manduca sexta*, PER immunofluorescent labeling was performed with a specifies-specific antibody raised against a 358 aa long *M. sexta* PER peptide containing the PAS-domain and 24 residues downstream (Wise et al., 2002). They used an additional staining method with *in situ* hybridization, but both approaches revealed nuclei of a conspicuous cell cluster in the lateral brain close to the medulla that consisted of 100-200 small neurons and is very reminiscent on the LN_1 cluster of the honey bee brain. Furthermore, the nuclei of the photoreceptor cells were stained in the compound eyes and they found round or longish nuclei of many glia cells throughout the brain, the antennal and optic lobes. Four large PER/corazonin/leu–enkephalin expressing neurosecretory cells, located to the pars lateralis in the dorsal brain, exhibited both nuclear and cytoplasmic PER staining. In contrast to the beetle, these neurosecretory cells were less intensively stained than the other cells with nuclear PER and there was also no daily cycling in PER abundance. In sum, the staining pattern of *ms*PER is highly similar to the *am*PER-positive staining in the honeybee brain. Nevertheless, there is one remarkable difference between the hawkmoth and the honeybee brain: the PER/PDF-positive LN_2 cell cluster is absent in the hawkmoth. This fits to observations of Homberg et al. (Homberg et al., 1991b) who found no PDF-positive neurons in the lateral brain of the hawkmoth and Sauman and Reppert (Sauman and Reppert, 1996), showing no co-expression of PER and PDF in the lateral brain of the silk moth.

There are also a few Lepidoptera species, in which no PER-positive staining occurred in the lateral brain such as the silkmoth, *Bombyx mori*, (Sehadová et al., 2004) and the Chinese tussar silkmoth, *A. pernyi*, (Sauman and Reppert, 1996). A recent study in the Mediterranean flour moth, *Ephestria kuehniella*, which is phylogenetically more at the basis of Lepidoptera as compared to the very divergent group of Macrolepidoptera to which *B. mori*, *A. pernyi*, *M. secta* and *D. plexippus* belong, showed PER-positive staining in hundreds of neurons in the lateral brain (Kobelková et al., 2015). This suggests that the ancestral lepidopteran clock possesses clock neurons in the lateral and dorsal brain and that during evolution the number of clock cells was reduced and clock neurons may have disappeared in certain regions of the brain. In *E. kuehniella* PER was found in the cytoplasm and nucleus of most PER-positive cells and in *M. sexta* nuclear PER staining was in hundreds of PER-positive cells as mentioned above. This stands in contrast to the purely cytoplasmic PER staining in *A. pernyi*, *B. mori* and *D. plexippus*. Nevertheless, PDF-positive clock cells in the lateral brain have so far not been reported for any of the Lepidoptera species. This indicates that the Lepidoptera clock differs neuroanatomically from the other insect clocks. These differences are most evident in *A. pernyi* and *D. plexippus*, species for which even an alternative circadian clock mechanism has been proposed (Sauman and Reppert, 1996; Zhu et al., 2008).

In various other insects, including firebrats, mayflies, damselflies, stoneflies, caddisflies, butterflies and blood-sucking bugs, PER-positive cells were revealed in the dorsal and lateral brain (Sauman et al., 2005; Vafopoulou et al., 2009; Závodská et al., 2003). In all these cases PER staining was purely cytoplasmic. Only in the blood-sucking bug, *Rhodnius prolixus,* eight neurons in the lateral brain were

co-labeled with anti-PDF (Vafopoulou et al., 2009). In all other insects no co-expression of PER and PDF occurred in the lateral cells. Nevertheless, PDF was present in a cell cluster close to lateral PER-positive neurons in the majority of them (Závodská et al., 2003). In these studies, different PER-antisera were applied in parallel that were either raised against different parts of *D. melanogaster* PER or against conserved PER sequences of the Chinese tussar silkmoth, *A. pernyi*. In *R. prolixus* the strongest staining was achieved with one antibody against a long piece of *Drosophila* PER (Vafopoulou et al., 2009), in the other insects one PER antibody against *A. pernyi* (Sauman and Reppert, 1996; Závodská et al., 2003) worked best. Unfortunately, no real control staining on animals without PER could be performed and the antisera could also not be pre-absorbed on the relevant species-specific PER sequences, because these were not known. Thus, it cannot be completely excluded, that the antisera cross-react with one of the many peptides/proteins present in neurosecretory cells.

In summary, the honeybee circadian clock shows striking similarities in location to the clocks of fruit flies, cockroaches, beetles and hawkmoths. In all these species, PER was found at least temporally in the nucleus, where it can exhibit its role as transcriptional repressor in the molecular feedback loop.

Lack of gated translocation from the cytoplasm to the nucleus

The strong PER-level oscillations in the neuronal clusters in bees provide additional evidence that these cell clusters are pivotal components of the circadian network of bees. Peak abundance of PER protein in the three neuronal clusters LN_1, LN_2, and DLN is in the late dark phase (see here also time course in Fuchikawa et al., 2017). In my experiments with *Drosophila* this was a time point of high PER abundance, too. The phase of cycling is consistent with that reported by Bloch et al. (Bloch et al., 2003) and it fits well to mRNA studies showing that *per* mRNA abundance is highest around midnight. Thereby a time lag between peak abundance of mRNA and Protein of approximately 6 hours is demonstrated, which is essential for the molecular clock mechanism (Hardin et al., 1992; Sumiyoshi et al., 2011). Interestingly, *am*PER staining was not detected in the cytoplasm. It could be that sampling with a higher time resolution or improvement in spatial resolution in microscopy reveals staining of cytoplasmic PER. Nevertheless, there is another explanation: unlike *Drosophila* PER which has been found also in the cytoplasm at certain times of the day (Shafer et al., 2002) there is no or very little accumulation of PER in the cytoplasm prior to its translocation into the nucleus in honey bees. This notion is consistent with a similar lack of cytoplasmic accumulation recently reported for the mammalian PER orthologue mPER2. Smyllie and co-authors (Smyllie et al., 2016) generated a knock-in mouse expressing a PER2::VENUS in vivo reporter that did not compromise the functioning of PER2 in cells of the mammalian suprachiasmatic nucleus (SCN). PER2::VENUS abundance in these mice varied rhythmically in the nuclei of SCN neurons, but was virtually absent from the cytoplasm at all time points. This apparent similarity of the bee with the mouse rather than with *Drosophila* adds to earlier evidence that its molecular clockwork is in many ways more similar to mammals than to *Drosophila* (Rubin et al., 2006). Like in mammals, the honey bee genome does not encode orthologues to TIM1 and CRY-d (insect Cry1), but rather encodes a mammalian-like CRY (CRY-

m/insect CRY2), that our IP (see Fuchikawa et al., 2017) confirms to interact with PER and repress the transcriptional activators CYC and CLK (Rubin et al., 2006; Weinstock et al., 2006; Yuan et al., 2007). Smyllie and co-authors (Smyllie et al., 2016) explained the contrasting behavior for the fly and mouse PER by the proteins having different hetero-dimerization partners (dTIM1 and mammalian-type CRY, respectively). Translocation from the cytoplasm to the nucleus was also not detected in most other insect species, in many of which PER-staining was always in the cytoplasm (Sauman and Reppert, 1996; Shao et al., 2006; Vafopoulou et al., 2009; Wen and Lee, 2008; Wise et al., 2002; Závodská et al., 2003). This raises the interesting hypothesis that the variation in nuclear or cytoplasmic location of PER staining may at least be partially explained by the variability in PER dimerization partners seen in different species (Tim1, Cry-m or both).

In summary, my studies provide a detailed characterization of the honey bee brain clock network and the first description of the circadian clock in solitary bees. This sets the stage for further comparison of the brain anatomy of solitary and social bees together with investigations on the interactions between circadian clock network and complex behaviors. Thereby we might gain new insight into the evolution of eusociality among insects. My results also reveal common neuroanatomical organization principles in insects. Similar analyses with specific antibodies for additional species are needed to better understand the conservation and evolutionary modifications in the clock network of various insect groups.

4.4. PDF network in bees (see also attached manuscript Beer et al., 2017)

My experiments lead to the assumption that PDF neurons in the honey bee brain fulfil all required anatomical prerequisites to transfer rhythmic signals to other brain regions, including those involved in regulation of locomotor activity, time associative memory and time-compensated sun-compass orientation. The PDF network in the honey bee is built by the LN_2, with somata positioned in the ventro-lateral brain and arborizations into virtually all brain regions. Their neurites are often accompanied by PER-positive glia cells and are in close proximity to the PER-positive LN_1, DLN and DN. The staining pattern in solitary bees is highly consistent and displays only minor differences to the PDF network in honey bees, which might indicate adaptations to social lifestyle of honey bees. Furthermore, the PDF network in bees shows similarities to PDF staining patterns in other insects, like for example the fruit fly *Drosophila melanogaster* (Diptera), the cockroach *Rhyparobia maderae* (Blattodea) and the cricket *Gryllus bimaculatus* (Orthoptera).

PDF fibers pass next to PER expressing neurons and glia cells

As described above the clock protein PER of the honey bee *Apis mellifera* is present in neurons in the lateral and dorsal brain, as well as in numerous glia cells throughout the brain and the optic lobes similar to the distribution in *D. melanogaster* (Fuchikawa et al., 2017; Siwicki et al., 1988; Zerr et al., 1990). Here, I show that fibers arising from the PDF-positive LN_2 come close to most other clusters of PER-expressing neurons, and are accompanied by PER-positive glia cells in most brain regions. This finding is similar to PDF immunostaining in *Drosophila melanogaster*, in which the PDF neurites from the PDF-positive LN_v are accompanied by PER-positive glia cells (Helfrich-Förster, 1995; Suh and Jackson, 2007) and come close to other PER-positive neurons in the lateral and dorsal brain (Helfrich-Förster et al., 2007b). It was further shown in *Drosophila,* that many of these PER-positive cells also express the PDF receptor (Hyun et al., 2005; Im and Taghert, 2010; Mertens et al., 2005; Shafer et al., 2008) and that PDF signaling strongly affects the oscillations of the other clock neurons and the fly's behavioral rhythmicity (Choi et al., 2009; Eck et al., 2016; Grima et al., 2004; Guo et al., 2014; Helfrich-Förster, 1998; Lin et al., 2004; Nitabach et al., 2006; Peng et al., 2003; Shafer and Taghert, 2009; Sheeba et al., 2008; Wu et al., 2008; Wülbeck et al., 2009; Yoshii et al., 2009a). The close proximity of PDF fibers and PER-positive glia in the honey bee is highly evocative. In *Drosophila,* it was shown that electrical manipulation of PER-positive glia cells causes arrhythmicity, suggesting that glia cells are involved in the clock network and important for rhythmic behavior (Jackson et al., 2015; Ng et al., 2011). The importance of PER-positive glia cells for circadian rhythms was also recently demonstrated in the mammalian circadian clock, the SCN (Brancaccio et al., 2017).

Comparison of PDF arborization pattern in the bee brain with that of other insects

PDF immunocytochemistry was performed in the brain of many insects including locusts, cockroaches, crickets, bugs, cicadas, flies and bees (Abdelsalam et al., 2008; Bloch et al., 2003; Helfrich-Förster and Homberg, 1993; Homberg et al., 1991a; Lee et al., 2009; Nässel et al., 1991, 1993; Sato et al.,

2002; Stengl and Homberg, 1994; Sumiyoshi et al., 2011; Weiss et al., 2009), with the most detailed description available for the cockroach *Rhyparobia maderae* (formerly *Leucophaea maderae*) (Homberg et al., 2003; Petri et al., 1995; Reischig and Stengl, 2002, 2003b; Reischig et al., 2004; Stengl and Homberg, 1994; Wei et al., 2010) and the fruit fly *D. melanogaster* (Helfrich-Förster, 1997, 2005b; Helfrich-Förster et al., 2007b). In all these species, with the exception of moths and butterflies (Homberg et al., 1991b; Wise et al., 2002), PDF-positive somata could be localized to the optic lobes or the lateral protocerebrum.

Number and size of the PDF neurons:

I found in social and solitary bees on average ~15 PDF-positive somata of different size, which are all located close to the anterior medulla. This number fits to the ~14 PDF somata reported previously for honey bees (Bloch et al., 2003; Sumiyoshi et al., 2011) and the 9-15 PDF somata found in bumble bees (Weiss et al., 2009). It also roughly fits to the number of ~ 12 PDF-positive somata located anteriorly of the medulla in the cockroach (aPDFMe neurons (Reischig and Stengl, 2003b)). The cockroach possesses additionally 8 PDF neurons with somata posterior of the medulla and many PDF neurons with small somata close to the dorsal and ventral lamina (Wei et al., 2010). Such PDF-positive somata do not exist in the bee brain. Furthermore, whereas the somata of the cockroach aPDFMe neurons are located at the ventromedial edge of the medulla, the bee PDF neurons are located at its dorsomedial edge and have a larger distance to the medulla than the cockroach aPDFMe.

In all species investigated so far the PDF somata vary in size (Bloch et al., 2003; Helfrich-Förster and Homberg, 1993; Malpel et al., 2002; Nässel et al., 1991, 1993; Reischig and Stengl, 2003b; Sumiyoshi et al., 2011; Wei et al., 2010). I observed one particularly large, strongly stained neuron that was localized anteriorly to the other PDF-positive neurons whereas middle-sized and smaller somata were usually located further posterior and closer to the PDF fiber network. In *D. melanogaster*, 4 neurons with a small soma (and weak staining) project towards the ipsilateral dorsal protocerebrum and 4 neurons with a large soma (and strong staining) have wide projections connecting the AMEs of both brain hemispheres and forming a network in a distal layer of the medulla in both brain hemispheres (Helfrich-Förster and Homberg, 1993). The cockroach aPDFMe neurons could be subdivided into three groups according to soma size and staining intensity (4 large, 4 medium-sized and 4 small neurons) by the means of light and electron microscopy and analysis in a 3-D computer model (Reischig and Stengl, 2003b; Wei et al., 2010). In the bee, it was not possible to unequivocally classify the somata into different size categories by the means of light microscopy. Further analysis of the PDF somata in 3-D reconstructions and their ultrastructure might clarify, if there are different size categories like in the cockroach. Nevertheless, as reported for the cockroach (Wei et al., 2010), I also observed one especially large neuron that was strongly stained and that was located anteriorly of the others.

In cockroaches and flies, the PDF neurons with large somata appear to show wide field arborizations that span the entire brain and connect both brain hemispheres (Helfrich-Förster et al., 2007b; Reischig and Stengl, 2003b; Reischig et al., 2004). Unfortunately, the PDF fibers completely overlapped already

close to their origin at the somata wherefore I could not assign fibers to individual neurons in neither honey bees nor solitary bees. Nonetheless, similar to cockroaches and flies the larger PDF neurons in the bee brain may arborize throughout the entire brain, whereas the ones with small soma may remain local within the ipsilateral hemisphere. To elucidate the projection pattern in the highly complex PDF neuron network of the bee one could apply neuron tracing methods such as injection of fluorescent dyes combined with immunofluorescent labeling of the PDF neurons to identify traced axons (Wouterlood, 2015). Another approach would be to investigate different developmental stages of the bee brain and describe the arborization pattern of the PDF cells progressively arising throughout development (see also chapter 4.5.).

Is there an accessory medulla (AME) in the bee?

The AME can be regarded as the most important communication center in the circadian network of insects, which additionally gets input from external Zeitgebers (environmental time cues). This small neuropil at the base of the medulla is typically characterized by a dense network of PDF-positive fibers. It is best characterized in the cockroach *R. maderae*, where it is densely innervated by PDF-positive and other peptidergic neurons that may be similarly engaged in the circadian clock (Petri et al., 1995; Reischig and Stengl, 2003b; Reischig et al., 2004). It is organized into a nodular core receiving photic input from the eye and into an internodular and peripheral neuropil involved in efferent output and coupling input from other clock neurons (Homberg et al., 2003; Petri et al., 1995). In *D. melanogaster*, the AME is less conspicuous, but as in the cockroach it receives dense input from the PDF-positive neurons as well as from the majority of other clock neurons and it is innervated by an extra retinal eye, the Hofbauer-Buchner eyelet (Helfrich-Förster et al., 2002; Hofbauer and Buchner, 1989; Malpel et al., 2002).

I identified a small neuropil at the base of the honey bee medulla which is even less noticeable than the AME of flies and is not densely innervated by PDF-positive fibers. Interestingly, an area with much higher density of PDF fibers was found proximally of the PDF neurons in front of the lobula that I called ALO. Thus, I hypothesize that the ALO is a more important communication center of the honey bee circadian clock neurons than the AME. A similar located PDF-rich network was also described for the bumble bee, *Bombus terrestris* (Weiss et al., 2009). In solitary bees, I observed the high density network of the ALO in the same position dorso-anterior of the lobula, too. Nevertheless, I did not confirm the location with neuropil staining with anti-HRP antibody like in the honey bee. I suggest that the bee ALO is not anatomically homologous to the AME of the cockroach or the fly. Nevertheless, the ALO of bees is located very close to the somata of the PER-positive LN_1. In *Drosophila,* several lateral neurons are in close vicinity of the AME and the majority of the other PER-positive clock neurons project into the AME. Additional studies are needed in order to determine whether the bee LN_1 and the other PER-positive clock neurons do also project into the ALO. The apparent neuroanatomical differences in the circadian clock center of bees and other insects may have its cause in development. In many insects the AME develops from the larval optic neuropil that is innervated by larval photoreceptors and serves as a common visual neuropil (Hagberg, 1986; Helfrich-Förster et al., 2002;

Schulz et al., 1984; Yasuyama and Meinertzhagen, 1999). During metamorphosis, the larval photoreceptors often degenerate and the larval optic neuropil transforms into the AME. In *Drosophila melanogaster*, the larval optic neuropil is already innervated by PDF-positive neurons (Wegener et al., 2004). These PDF cells serve also as interneurons mediating photophobic behavior, which is important for the orientation of the freely moving larvae (Mazzoni et al., 2005). Thus, the AME and its precursor are strongly associated with photoreception. Unlike the fly larvae, the honey bee (as well as the solitary bee) larvae does not need to search for food based on visual cues but is nursed or receives food provisions before larval development (solitary bee) and remains in the dark. Thus, a larval optic neuropil that receives photoreceptor input might be missing and the PDF neurons do not have to serve as photoreceptor interneurons. As a result, there was no need to develop a PDF-rich AME that is strongly associated with extra retinal photoreceptors. Instead, bees appear to have developed an alternative circadian communication center in the ALO. The ALO is located between the AME and the lobula valley tract (LVT), and even in cockroaches, the PDF fibers pass this region anterior to the lobula. The only difference is that in cockroaches the PDF fibers have the densest arborizations in the AME, whereas in bees they have these in the ALO. I do not suggest that bees do not possess an AME, but I argue that, during development, a close association with photoreceptors is lacking and consequently a dense innervation by PDF fibers is missing. Therefore, I performed developmental studies to clarify how the PDF neurons and their arborizations look during larval and pupal stages (see discussion below).

Connections of the PDF neurons with higher integration centers in the brain

Both time memory and sun-compass navigation require that information about time of day is conveyed to brain structures responsible for memory and sun-compass orientation. Below I will discuss whether PDF neurons fulfil the anatomical criteria to provide such an input.

Connections between PDF neurons and memory centers in the mushroom bodies

Honey bees are excellent learners, quickly forming associations between stimuli of different sensory modalities (Giurfa, 2007) and showing various forms of conceptual learning (Giurfa et al., 2001) that are largely mediated by their highly developed mushroom bodies. The calyces of the mushroom bodies (MCA and LCA) are large and receive input from olfactory and visual cues (Gronenberg and Lopez-Riquelme, 2004), whereas the vertical lobes are thought to be the main output regions of the mushroom bodies (Menzel, 2012). I found that PDF neurons do not invade the mushroom body neuropils, but rather wrap their various parts. The densest network of PDF fibers is found ventrally to the lateral and median calyces of the mushroom bodies, but also the vertical, median lobes and the peduncle are surrounded by varicose neurites of PDF neurons. For example, the PDF fibers form rings around the vertical lobe and the peduncle. Since PDF is most likely stored in the varicosities and released from there (and other parts of the neurite) in a paracrine manner, it likely can convey time-of-day information to the mushroom bodies, and this appears possible in many places.

Connections between PDF neurons and the sun-compass pathway

The neuronal basis and mechanisms underlying sun-compass orientation have been investigated in detail in locusts (reviewed by (Homberg, 2015; Homberg et al., 2011). Two major pathways that transfer sun compass signals to the central complex - a prominent anterior one and a less striking posterior one – have been described (el Jundi and Homberg, 2010). Recently, three papers have been published indicating that at least the anterior polarization vision pathway is conserved between honey bees, locusts, bumble bees and ants (Held et al., 2016; Schmitt et al., 2016; Zeller et al., 2015). The sun-compass pathway receives skylight polarization input from a specialized area of the compound eye, the dorsal rim area (DRA). Photoreceptors in this area project through the dorsal lamina and terminate in the dorsal rim area of the medulla (MEDRA). The MEDRA is innervated by transmedulla neurons that carry the polarized light information through the serpentine layer of the medulla and then via the anterior optic tract to the anterior optic tubercle (Zeller et al., 2015). From there, interneurons form a connection to the lateral and medial bulbs (MBU and LBU) (Mota et al., 2011; Zeller et al., 2015). In the bulbs, they form conspicuous large synapses with GABA-ergic tangential neurons of the central body's lower division (CBL) (Held et al., 2016). These neurons are then connected to the protocerebral bridge of the central complex which holds a topographic representation of zenithal polarization angles (Heinze and Homberg, 2007, 2008; Heinze et al., 2009). The possible posterior polarized-light input pathway starts also from the serpentine layer of the medulla and ends in the protocerebral bridge; but it passes via the AME and the POC to the POTU that locate adjacent of the most lateral endings of the protocerebral bridge. Output neurons from the protocerebral bridge project to the lateral accessory lobe (LAL (Heinze et al., 2009; el Jundi and Homberg, 2010; el Jundi et al., 2010)) and finally, polarization information is send via descending neurons to thoracic motor control centers (Träger and Homberg, 2011). In honey bees, the pathways within the central complex as well as the output pathway to the lateral accessory lobe are not yet clarified, but are likely to be similar.

I found PDF-positive fibers (1) in the DRA, (2) between the DRA and MEDRA and (3) around the MEDRA. These fibers extend along the projections of the dorsal rim photoreceptors that run into the MEDRA. My results are in line with the data of Zeller and co-authors (Zeller et al., 2015), who combined PDF immunofluorescence with tracing of sky compass pathways to the MEDRA that is surrounded by PDF fibers. They further show that the PDF fibers in the serpentine layer of the medulla overlap with the pathway of the transmedulla neurons that run via the anterior optic tract into the central brain. Consistent with their observations, I did not find any PDF-positive fibers in the anterior optic tract and the anterior optic tubercle, but I saw a dense PDF fiber network directly behind the anterior optic tubercle. In addition, I observed a ring of PDF fibers around the vertical lobes, exactly at the location where the interneurons from the anterior optic tubercles take their path toward the bulbs (MBU and LBU). The bulbs themselves are not innervated by PDF fibers, but again, PDF varicosities are very close to them. There was also a dense PDF network in the median accessory lobe (MAL), a brain area that seems to be associated with the central complex (Homberg, 1991) and PDF fibers were in an area that resembles the putative lateral accessory lobe (LAL). The boundaries of the honey bee LAL, are less well defined and therefore I cannot be sure, whether the PDF fibers also enter the LAL, or just pass close to it. Concerning the possible posterior polarized-light input pathway, I found

PDF fibers that leave the POC close to the POTU and may innervate them. Thus, here might be a direct input from the PDF neurons.

In summary, I found PDF fibers in close vicinity to the sun-compass pathway, not only on its input side to the central complex, but also on its putative output pathway. Thus, a rhythmic paracrine release of PDF in several places can potentially convey time-of-day information to the sun-compass network. Furthermore, the activity control center resides in the LAL (as described above in the sun-compass pathway) and circadian release of PDF may here be directly conveyed to regulation of the circadian locomotion output behavior.

PDF neuron network displays complexity differences between social and solitary bees

The network build by the PDF fibers in bee brains is highly complex in comparison to other insects. There are 7 commissures innervated by PDF neurons in the bee, while representatives of Diptera, Hemiptera and Orthoptera have only 2-3 commissures and Blattaria have 3-4 (Homberg et al., 1991a; Sehadová et al., 2003; Stengl and Homberg, 1994; Závodská et al., 2003). This might hint to a stronger integration of time information into the regulation of behavioral processes in bees, but it has to be noted here that the PDF network is characterized only in few species so far and functional studies are largely missing. Further studies will show if this assumption is correct. Other parts of the PDF network in bees show strong similarity to the PDF staining pattern in other insects like the medulla fiber fan and the LVT, which were also described in cockroaches (Wei et al., 2010), whereby the “loop” of the LVT at the ventro-medial edge of the LO seems to be characteristic for bees. In the neuroanatomical comparison of the two bee species, I observed that the LVT as well as the POC in the solitary bee are not as densely innervated by PDF fibers as in the honey bee. As these areas are involved in transmission of visual inputs to centers of higher brain functions it might be that there is a difference in integration or processing of visual signals in the circadian system of social and solitary bees.

There are studies indicating that at least in case of learning and memory behavior and the associated brain anatomy there seems to be a difference in bees with either social or solitary lifestyle. Neuroanatomical studies in a facultative social living bee (*Megalopa genalis*) have shown that the social context can cause alternate brain development (Smith et al., 2010). *Megalopta* queens exhibit a significantly larger MB volume than workers or solitary reproductives. Two behavioral studies comparing learning performance of social and solitary bees demonstrate that the two bee types learn differently (Anfora et al., 2010; Dukas and Real, 1991). Anfora and co-authors propose even a fundamental restructuring of the central nervous system during evolution due to selective pressure associated with sociality after finding lateralization in behavior and electrophysiology in honey bees, but not in solitary bees. The PDF network in bees could have underwent a similar restructuring during evolution. Therefore, it will be interesting to investigate the involvement of the circadian system in complex behavior like division of labor, dance communication, sun-compass orientation, and time memory in social compared to solitary bees. These functional studies may give further credence to this hypothesis.

One behavior, which is exclusively associated to the lifestyle of social bees, is their dance language communication. Forager honey bees inform their nest mates in their “waggle dance” about direction and distance of available food (von Frisch, 1965). Sound and airflow signals generated by wing and abdominal vibrations during the dance are important for this communication, but the decoding mechanism and integration of sun-compass and gravity information (because the bees dance on the vertically positioned comb) is poorly understood. Bees need to consult the circadian system to communicate the position of floral resources, which their nest mates will then find by the means of time compensated sun-compass orientation (Beier and Lindauer, 1970; Brockmann and Robinson, 2007). Brain structures involved in sensory input or control of “waggle dance” behavior might be stronger innervated by clock neurons in honey bees than in solitary bees, which do not communicate this way (Greenfield, 2002). Brockmann and Robinson (Brockmann and Robinson, 2007) presented tracing experiments of neurons involved in sensory input pathways associated with dance language communication in the honey bee brain. These sensory systems were (1) neurons of the DRA, which are part of the sun-compass input pathway (Homberg et al., 2011; el Jundi et al., 2014; Zeller et al., 2015), (2) neurons of the neck hair plates, which are required to transform sun-compass information to gravity based information, when they dance on the comb (Ai and Hagio, 2013; Lindauer and Nedel, 1959), and (3) neurons from the antennal joint hair sensilla and the (4) Johnston’s organ, perceiving information on direction and distance via mechanostimulation by abdominal and wing vibrations (Ai et al., 2007, 2009; Michelsen et al., 1986; Rohrseitz and Tautz, 1999). Another factor influencing dance language communication is optic flow (Esch et al., 2001), but there is no distinct region, which perceives optic flow information in the honey bee and tracing experiments were therefore not practicable. Brockmann and Robinson (Brockmann and Robinson, 2007) found, consistent with other studies (Zeller et al., 2015), that neurons of the DRA projected into the MEDRA, while neurons conveying gravity based information into the SEG and mechanosensory neurons into the SEG, deutocerebrum and posterior protocerebrum (see also detailed studies confirming these results: (Ai and Hagio, 2013; Ai et al., 2007, 2009)). Unfortunately, it was not possible to identify dance language specific input pathways, because the sensory pathways described above are not unique to honey bees, instead path integration might be differently adapted in evolution of eusociality (Ai, 2013; Brockmann and Robinson, 2007). This would mean that there is not necessarily a neuroanatomical difference between dance language associated brain structures in social and solitary bees. This assumption fits to the highly similar PDF staining patterns in brains of social and solitary bees. I found neuron arborizations in the polarized light input pathway of the sun-compass as well as in the SEG, the deutocerebrum and in the posterior protocerebrum in both bee species. Moreover there is in both bees a dense PDF network surrounding the mushroom bodies and central complex, which may represent the brain center integrating all sensory information required for dance language communication and transferring it to motor neurons (Barron et al., 2012). Nevertheless, I saw a difference in PDF innervation density in the posterior protocerebrum, especially the POC, between the two investigated bee types. If this is indeed associated with an adaptation of the circadian system integration into dance language of honey bees cannot be answered at the moment. For example, the POC in the ant *Camponotus floridanus* is similarly sparsely innervated like in the solitary bee (Janina Kay, personal communication). This ant lives eusocially, but is only distantly related to honey bees,

which resulted in different recruiting communication, primarily based on mechanical and chemical stimuli, and these ants have not been shown to use dance language or sun-compass orientation (Hölldobler, 1995; Traniello, 1977). There is also no report that solitary bees use sun-compass orientation or purely rely on landmark orientation. Therefore it is unclear if the PDF fiber density in the posterior protocerebrum, where also the second input pathway of the sun-compass system lies (el Jundi and Homberg, 2010), is associated with sun-compass navigation or dance language communication. Functional studies and further detailed anatomical investigations in several bees with different (social) lifestyles are needed.

Another striking difference between the PDF networks of honey bee and mason bee was the densely innervated knot like structure in the lamina of mason bees. It resembles the dense innervation in the DRA, but is not at the right location. A similar structure has been described in the circadian system of the beetle (Frisch et al., 1996). Here it was defined as extra retinal photoreceptor, called lamina organ, and was innervated by PER-positive neuron fibers, but not labeled with anti-PDH antibody. Spaethe and Briscoe (Spaethe and Briscoe, 2005) describe a UV-opsin expressing photoreceptor in the proximal lamina of bumble bees, where also PER is localized. They speculate that this area of the proximal lamina in bumble bees serves a similar function like the lamina organ in other insects. Nevertheless, the opsin staining pattern in the lamina of bumble bees does not resemble the pattern of this conspicuous knot like structure in the mason bee. Therefore, I cannot be sure of the nature of this structure. The size of the knot like structure is reminiscent of a cell body, but staining intensity does not match to the other PDF somata and rather resemble fiber structures (this is also the case in higher magnification pictures, not shown in this thesis). In cockroach, cricket, locust, phasmid, and cicada additional PDF cell bodies reciting in the lamina have been described (Homberg et al., 1991a; Sato et al., 2002; Wei et al., 2010) and in most cases these cell bodies are grouped in dorsal and ventral clusters (Homberg et al., 1991a; Wei et al., 2010). The position of the dorsal lamina PDF cluster in the proximal posterior lamina region coincides with the location of the knot like PDF fiber structure I found. Therefore co-staining with DAPI and neuronal somata marking HRP may clarify if this structure is indeed part of the fiber network or rather lamina PDF neuron cell bodies. This raises the question if this difference in the PDF network structure in social and solitary bees may indicate a progressive loss of the lamina PDF neurons in bee evolution.

In sum, the detailed neuroanatomical description of PDF fibers together with the description of PER staining pattern in bee brains provides the best description of the bee circadian network available so far. PDF fibers stemming from the LN_2 clock neurons arborize extensively throughout the bee brain and reach other clock cells. Further arborizations innervate the optic lobes that receive visual input and come in close proximity to areas in the central brain that are important for memory formation, locomotion control and sun-compass orientation. I conclude that the neuroanatomical structure of the PDF network in bees is excellently positioned for conveying time-of-day information from the LN_2 to brain centers involved in various clock output functions. These include locomotor activity, sun-compass orientation and time-associative learning. Small anatomical differences in the arborization complexity between social and solitary bees may be adaptations to the different lifestyles. Studies on

the interplay between the circadian clock and complex behaviors such as division of labor, dance communication, sun-compass orientation, and time memory combined with the description of clock controlled behavior in solitary bees, which is largely unattended so far, may prove this assumption.

4.5. Development of the circadian clock in social and solitary bees

In a neuroanatomical study with brains of *A. mellifera* in different larval and pupal stages I can show that PDF network in honey bee brain grows and gains complexity throughout development. There are striking similarities to larval development in another holometabolous insect, *D. melanogaster*. In both insects, connections to the dorsal brain are formed first and later contralateral and widespread arborizations. Comparing ontogeny of circadian rhythms in locomotor activity of adult *A. mellifera* and *O. bicornis* I demonstrate that the honey bee has a different timing in development of the circadian system and newly emerged bees seem to lack a fully matured clock. I further argue that this difference in post-emergence clock development in social and solitary bees can also be demonstrated on the neuroanatomical level via staining experiments with the neuropeptide PDF as key component in the bee clock. Based on my studies with social and solitary bees I raise the hypothesis that premature emergence may have evolved in association with sociality.

Comparison of PDF neuron development in honey bees and other insects

PDF network development in the brain of *A. mellifera* and *D. melanogaster*, two holometabololous insects, is highly similar. Like in *Drosophila* few PDF-positive neurons are already present in a very early larval instar (*Drosophila*: L1 of 3 instars in total, *Apis*: L3 of 5 larval instars) and the number increases in later larval stages and throughout pupal development (Helfrich-Förster, 1997). Unlike in *Drosophila* it was not possible to classify PDF neurons into different size categories (Kolbe, 2013) nor could I define sub-cluster categories in honey bees, but in both animals the early developing neurons project dorsally and then laterally into the dorsal parts of the central brain and the optic lobe. Furthermore, in both holometabolous insects the somata of the PDF-positive neurons stay close to the lateral central brain, which is not the case in the hemimetabolous locust larval brain, where cell bodies migrate during development into the optic lobe and two additional PDF cell cluster are formed in the lamina (Helfrich-Förster, 1997; Homberg and Prakash, 1996). Furthermore, fruit flies and locusts have additional PDF neurons (in *Drosophila* these are called PDFTri (PDF-tritocerebrum) neurons) in late pre-mature development, which are reduced during last metamorphosis or early adulthood. On the contrary, honey bees like blood sucking bug possess a highly similar PDF-network before and after their last metamorphosis (Vafopoulou et al., 2009). Contralateral projections and all main connections to the different brain areas are already established in bee pupal stage 1, while in *Drosophila* contralateral projections are formed only in later pupal stages (Helfrich-Förster, 1997). The connections to the dorsal brain may be already of importance for the integration of time information into olfactory learning processed in the calyces of the mushroom bodies (Gronenberg and López-Riquelme, 2004). Additionally, centers for non-visual entrainment like the temperature entrainment may be contacted by the PDF fibers. At least in *Drosophila* the center for temperature entrainment lies in the dorsal brain (Miyasako et al., 2007; Tomioka et al., 2008), and similarly temperature entrainment may be possible in bee larvae. Whether this ability is of biological relevance in honey bees, as they are raised in the constantly thermoregulated hive, is debatable, but solitary bee species have been shown to gain fitness, when raised in daily temperature cycles (Rinehart et al., 2013; Yocum et al.,

2012). Larvae do not need integration of the circadian system into more complex behavior like sun-compass orientation and PDF fibers reach brain centers for controlling this behavior (especially central complex) (el Jundi and Homberg, 2010; Träger and Homberg, 2011) only later in development . Newly developing neurons intensify the existing fiber bundles and grow into the same paths as already existing neurons. This is especially prominent at the dense fiber network called anteriolobular PDF hub (ALO) very close to the somata. In the ALO, all PDF fibers intermingle and singular neurite tracing is not possible even in larvae brains. In *Drosophila* the larval photoreceptor, called Bolwig's organ (a derived form of stemmata in other insects) develops into the extra retinal photoreceptor, the HB-eyelet, which projects directly into the accessory medulla (AME) (Hofbauer and Buchner, 1989; Melzer and Paulus, 1990; Yasuyama and Meinertzhagen, 1999). In the AME the terminals overlap with arborizations of the PDF-positive neurons (Helfrich-Förster et al., 2002). Both Bolwig's organ and HB-eyelet have been shown to be involved in the entrainment of the circadian clock (Helfrich-Förster, 2005b). Aculeate hymenoptera like *A. mellifera* do not have stemmata (Gilbert, 1994) and therefore the AME of *Drosophila* and the ALO in the honey bee are likely not homologues (see also chapter 4.4. and attached manuscript Beer et al., 2017). It is therefore not surprising that the ALO in the bee larva is less associated with the ME than rather with the LO, like it is in the adult bee brain.

The development of the circadian system is timed differently in social and solitary bees

In the following, I will firstly discuss behavioral studies on the ontogeny of circadian rhythms in the post-emergence development of social (*A. mellifera*) and solitary (*O. bicornis*) bees. Honeybees develop circadian behavior later than solitary bees, which might be due to a not fully matured circadian system. In the second part, I will discuss that another readout for a potential difference in maturation of the circadian clock may be immuno-fluorescent staining of PDF in the brains of different bee types at different ages.

Timing in developing circadian behavior

The social lifestyle of honey bees has an impact on the behavior governed by the endogenous circadian clock: whereas younger nurse bees exhibit no circadian locomotor activity, older forager bees show strong circadian activity rhythms (Bloch and Robinson, 2001; Moore et al., 1998). Nevertheless, both, nurse and forager bees seem to have a functional circadian clock, because levels of the central clock protein PER and of the neuropeptide PDF cycle in a daily manner (see Fuchikawa et al., 2017 and Beer et al., 2017). Moreover, the arrhythmic behavior in nurse bees is context dependent, because nurse-aged bees are able to display rhythmic activity when they are not nursing. This is the case for example when they are interacting with forager bees or when they are deprived of the brood (Bloch and Robinson, 2001; Crailsheim et al., 1996, 1999). Apparently the circadian locomotor activity in bees is a highly plastic behavior and earlier studies suggested an ontogeny of circadian rhythms at the age of ~ 1 week in the laboratory and ~1-3 weeks in nature (Moore et al., 1998; Toma et al., 2000). Nevertheless, in 2012 Eban-Rothschild and co-authors found out that two

day old bees raised in the colony can already exhibit circadian locomotor rhythms (Eban-Rothschild et al., 2012). They reported furthermore, that young honey bees raised outside of the colony environment display a delayed onset or complete lack of circadian rhythms in locomotor activity and concluded that the social environment of a colony promotes the normal development of the circadian system in young honey bees. This is in line with my findings in my first publication, in which young honey bees exhibit significantly earlier daily rhythmicity in their behavior, when they have social contact to a mini colony (see Beer et al., 2016). Moreover locomotor activity profiles in premature forager bees raised in the laboratory differ from those in naturally developed forager bees by showing less power of rhythmicity (Eban-Rothschild et al., 2012 and (Beer et al., 2016).

Recently it has been shown that raising temperature plays a pivotal role in the ontogeny of circadian locomotor rhythms as well. Bees kept at 35°C for the first 24 h of their life display rhythms already at the age of 1-2 days while the bees kept at 25°C need a few days longer (Giannoni-Guzman, 2016; Nagari et al., 2017b). This is not surprising, because the correct temperature is important for a normal brain development (Groh et al., 2004; Jones et al., 2005; Tautz et al., 2003). The temperature in the core of a hive, where the newly emerged bees reside, is 32–36°C (Kleinhenz, 2003; Kronenberg and Heller, 1982; Simpson, 1961; Stabentheiner et al., 2010), but the typical temperature in the laboratory during activity monitoring of the young bees was below the core hive temperature at ~26°C (Bloch et al., 2001; Eban-Rothschild et al., 2012; Toma et al., 2000). Although the temperature I used my circadian rhythm ontogeny experiment in honey bees was 30°C, which is better comparable with the core hive temperature, the brood comb, from which I took the newly emerged bees, was stored at 20°C overnight. These few hours of low temperature may have delayed normal development of the circadian system in the bees. It would be interesting to investigate the circadian rhythmicity onset in locomotor activity of individual bees with and without contact to a mini-colony at a temperature close to the core hive temperature (and newly emerged bees directly taken from the outdoor hive) and thereby imitate hive conditions in the laboratory.

Nevertheless locomotor activity studies with newly emerged honey bees indicate that the circadian system is not fully matured in these bees (Bloch et al., 2001; Eban-Rothschild et al., 2012; Giannoni-Guzman, 2016; Moore et al., 1998; Toma et al., 2000) (see also Beer et al., 2016) and maturation speed depends on at least two factors: temperature and colony environment. Unlike the honey bee, which emerges in the safety of a hive community, the solitary bee has to cope with daily environmental changes. Therefore, a fully developed circadian system is essential for their survival and fitting to my hypothesis that almost all solitary bees in my experiments displayed strong circadian rhythms right after their emergence. Fitting to this assumption the fruit fly *D. melanogaster,* which is exposed to environmental changes from early development on, displays locomotor activity rhythms from first instar larvae on (Sehgal et al., 1992) and clock protein cycle already in the larvae (Kaneko et al., 1997).

Studying anatomical differences in maturation of the circadian system in solitary and social bee brains with anti-PDF staining

There is also evidence of maturation of the circadian system in honey bees on the neuroanatomical and physiological level. Bloch and co-authors report that the number of PER-positive cells in the brain of foragers is higher than in the brain of nurses (Bloch et al., 2003). Likewise *per* mRNA levels are higher in forager brains and show circadian cycling, while *per* expression in nurse bee brains exhibit no rhythmic expression on the transcriptional level (Toma et al., 2000). Moreover although the PDF neuropeptide is released rhythmically in nurse bee brains PDF levels in different brain areas cycle highly synchronous in nurse bees, while in forager bees phases differ between brain areas (see attached manuscript Beer et al., 2017). And finally there are some indications in preliminary studies that cell number of PDF-positive cells seems to be higher in forager brains compared to nurse brains (Kolbe, 2013). It is not clear if the difference in cycling in different brain areas is dependent on development or on task. Nevertheless maturation of the circadian system might correlate with the age dependent polyethism that honey bees display: the transition from younger nurse bees to older forager bees is accompanied by the integration of time-of-day information into foraging behavior and time compensated sun-compass orientation (Beier and Lindauer, 1970). PDF neurons provide with their widespread arborization network in the bee brain the perfect infrastructure for conveying time-of-day information to the brain regions involved in time-memory and sun-compass navigation and indeed the PDF neurites overlap with input and output pathway of the sun-compass network (Zeller et al., 2015) (see also attached manuscript Beer et al., 2017). There are even other physiological indices that the honey bee brain matures further during adulthood. Electrophysiological responses of the Johnston's organ, which receives mechanical stimuli important for dance language communication in honey bees, are only detectable in forager bees, but not in hive bees (Tsujiuchi et al., 2007).

Functional studies via genetic manipulation of clock genes has not yet been successful in bees, but one study with *pdf* knock down crickets presented interesting parallels to behavior and clock gene expression in nurse bees. A *pdf* knock down in the cricket *Gryllus bimaculatus* caused a loss of power of rhythmicity in locomotor activity and crickets displayed less preference for activity during the night (Hassaneen et al., 2011). The pdf mRNA levels in knock down crickets were overall lower, the cycling in mRNA levels was abolished and the immunofluorescent signal in anti-PDF staining was reduced. This is reminiscent of the lower power of rhythmicity in young bees, the smaller amount of overall *per*-mRNA, the lack of cycling levels of *per*-mRNA and the lower PDF cell number in young nurse bees compared to forager bees. In *Drosophila* it has been shown that a certain subset of the PDF-positive neurons is sufficient to maintain rhythmicity in locomotor activity in constant dark conditions (Shafer and Taghert, 2009) which might explain that young bees with less PDF neurons are able to display circadian rhythmicity. Another function of PDF in the honey bee might be the coordination of different entrainment centers in the brain. PDF in the fruit fly is important for adjusting period and phase of rhythms and entrainment by light- and temperature-cycles displays synergistic effects (Yoshii et al., 2009b, 2009a). The cells involved in temperature entrainment reside in the dorsal brain of *Drosophila*, while the light-entrainment center is in the lateral brain and studies with pdf^{01} mutant flies demonstrated a coupling function of PDF between the two entrainment centers (Miyasako et al., 2007;

Tomioka et al., 2008). A similar function of PDF in coupling different entrainment centers in the brain of the honey bee could be possible. Young honey bees reside in the dark hive and do not need to integrate entrainment via light-dark cycles, but foragers need to process several environmental time cues. The fact that social entrainment cues for young nest bees seem to be more important than light give further credence to this hypothesis (Fuchikawa et al., 2016). This does not mean that the same hierarchy of synchronizing cues is not possible in older bees. I showed in my first publication (see Beer et al., 2016) that under certain conditions (no free flying) social cues are more important than light for synchronizing locomotor activity rhythms also in forager-aged bees. It might just simply be that the coupling of different entrainment centers in older bees improves entrainment strength or speed via different environmental factors. The PDF cycling with different phases in different areas in the forager brain is not in line with these assumptions, but I do not know if this phenomenon is caused by development of the PDF network or if it is task related. In preliminary studies with 1 week old (a typical age for nursing behavior in honey bees) *O. bicornis* I counted a similar number of PDF-positive neurons (~14 cells) like in honey bee forager brains. Nevertheless, studies in which I sample of complete aging time lines of honey bee and mason bee brains are still needed to harden my hypothesis of differential timing in clock development in the two bee species. There are many indications also on the neuroanatomical level that the honey bee circadian clock matures with age. PDF seems to be a good candidate to investigate differences in maturation of the circadian system in social and solitary bees.

The evolutionary advantage of a social clock

Sociality has evolved in multiple groups, whether mammals, insects or even microorganisms, because of evolutionary benefits from group hunting, development of resistance against disease or predators and increased reproductive success (Alexander, 1974; Crespi, 2001). Recently a study on groups of female baboons could prove that there is a direct correlation between social lifestyle and infant survival (Silk et al., 2003). Another study discovered that even normally solitary living bees developed traits like specialization and division of labor typically associated with sociality in forced social cooperation (Jeanson et al., 2008). The bees benefit from it, because in cooperation they were able to build deeper nests and could guard the excavation site at the same time. In case of the honey bee, improved colony defense is one clear benefit of sociality, but also the vast amount of offspring production in a colony (Breed et al., 1990). The associated brood care behavior evolved along with adaptation in the circadian system, and nurses can therefore be active around the clock with no ill effects (Bloch and Grozinger, 2011; Bloch and Robinson, 2001; Bloch et al., 2013; Moore et al., 1998). I propose another adaptation, namely that the development of the circadian clock is timed differently in social and solitary bees. The honey bee's time point of emergence is advanced in comparison to the solitary bee emergence. This shortened pre-emergence development could evolve because the social environment enabled honey bees to survive without a fully matured circadian system. The newly emerged honey bee can trade off certain functions of the circadian clock associated with foraging behavior and dance language or time-compensated sun-compass orientation and nevertheless fulfill

other functions in the colony a fully matured circadian system is not needed for. This might not be an evolutionary benefit for the individual bee, but it is certainly for the colony. Neuroanatomical findings in social and solitary bees lay further credence to this suggestion (Withers et al., 2008). Withers and co-authors measured the proportions of mushroom bodies compared to Kenyon cell bodies in *Apis mellifera* and *Osmia lignaria* and found that proportions in newly emerged *Osmia* are just as high as in foraging honey bees. Furthermore, the proportions do not change during aging in *Osmia*, while this is the case in *Apis*.

Interestingly the honey bee clock has many features which are typical for the mammalian and the human clock. Besides the mammalian type *cryptochrome* as part of the central TTFL (Rubin et al., 2006; Weinstock et al., 2006), honey bees exhibit a phase response curve similar to vertebrates (Aschoff and Pohl, 1978; Ludin et al., 2012). Like these they can only shift their activity a few hours per day and experience altered time perception similar to “jet lag” (Cheeseman et al., 2012; Ludin et al., 2012). In my studies I show another feature reminding of the human clock: Honey bees seem to lack a fully matured circadian clock right after emergence, which has been proposed also in previous studies (Eban-Rothschild et al., 2012; Meshi and Bloch, 2007) (see also Beer et al., 2016) just as human infants do. Some neurons of the human clock develop postnatally (Swaab, 1995) and rhythms in sleep wake cycles and hormone production shape at the age of 2 months (Kleitman and Engelmann, 1953; Rivkees, 2003). The social community in honey bee as well as human society provides protection to the offspring born with a partially undeveloped clock. Studying the eusocial and the solitary bees together offers the unique possibility to compare the impact of sociality on the development and structure of the endogenous clock.

In summary, I could show that the ontogeny of circadian locomotor rhythms in *A. mellifera* and *O. bicornis* is timed differently in their post-emergence development. Honey bees seem to emerge with a not fully matured circadian system. I discussed several indications that this hypothesis could be affirmed also on the neuroanatomical level. Furthermore, I showed that the PDF network in honey bee brains grows in neuron number and fiber network complexity throughout pre-emergence development. PDF staining in emerged bees of different ages of social and solitary bees could give evidence of differential timing in post-emergence clock neuron development, too. With the progress in the development of genetic tools and in sequencing of different species evo-devo studies gain increasing potential to contribute to establishing models of regulatory networks in evolution of sociality (reviewed in (Bloch and Grozinger, 2011; Robinson et al., 2005; Toth and Robinson, 2007)). Whether in evolution of sociality basic restructuring occurred on molecular and/or anatomical level or if rather small changes in key pathways occurred is a question that remains to be elucidated (Bloch and Grozinger, 2011). My comparison of the circadian system of social and solitary bees at the different levels of behavior, neuroanatomy and development may give better insight on mechanisms in social evolution in bees.

5. References

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6. Appendix

6.1. Supplemental figures from joint study with Mariela Schenk and me contributing equally

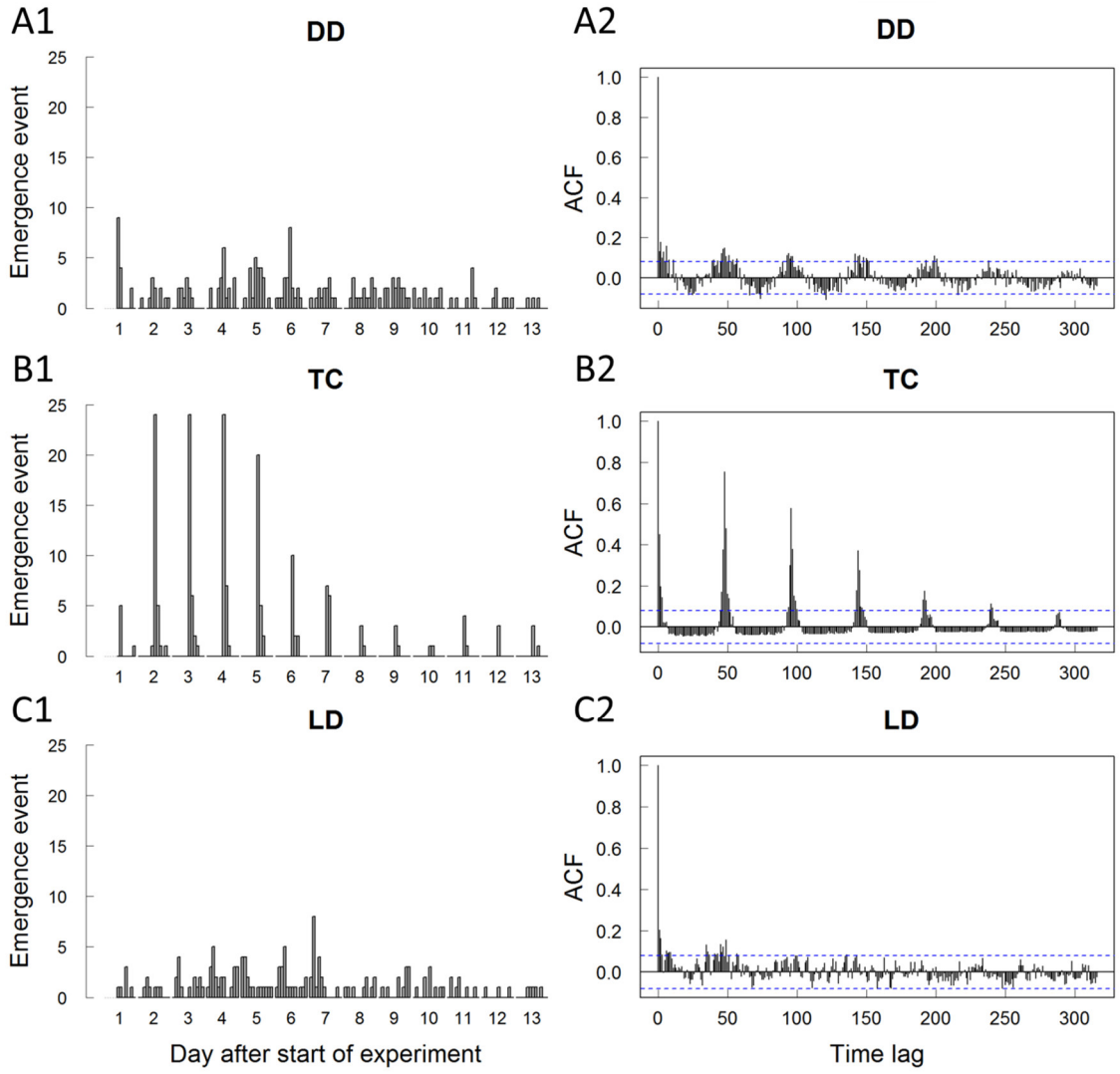

Figure S 1: Analysis of rhythmicity in emergence under different environmental conditions (joint study with Mariela Schenk).
Shown are the raw data of the emergence events throughout 13 days with a time bin of 2 hours (A1-C1) and the associated autocorrelation function for the emergence events with a time bin of 30 minutes, with 0.05% confidence interval (A2-C2). Graphs are shown separately for treatments (Constant darkness (DD): A1-A2; Temperature cycle (TC): B1-B2; Light-darkness cycle (LD): C1-C2).

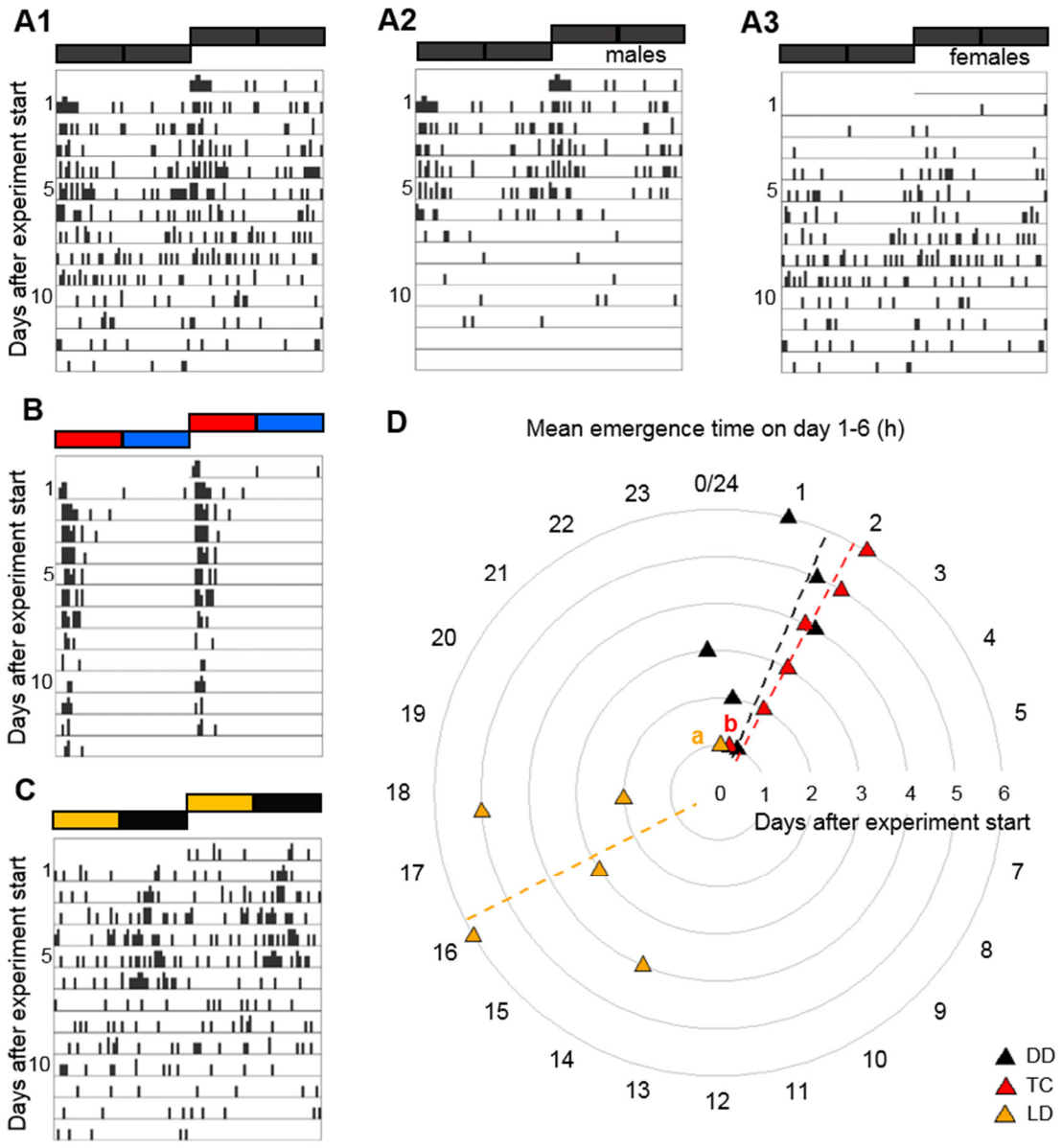

Figure S 2: Comparison of FRP between males and females and emergence activity patterns under different environmental conditions (joint study with Mariela Schenk).
Double plotted emergence actograms of test bees in constant conditions (DD) of the whole population (**A1**) and males (**A2**) and females (**A3**) respectively. The emergence events of all bees are plotted for the treatments with temperature cycles (TC) in **B** and light-darkness cycles (LD) in **C**. Emergence events in actograms are plotted in data bins of 30 minutes for the days 1 to 13 after the experiment start and environmental bars depict the different conditions of the experiments (black/black: constant conditions; red/blue: 12h high temperature/12h low temperature; yellow/black: 12h light/12h darkness). **D** shows the phase of mean emergence events on day 1-6 for the different treatments (DD, TC, LD), which indicate a differential phase relationship for LD compared to the other treatments. Circular axis: "time of the day" after start of the experiment. Watson U^2 test provides significant difference in emergence phase on day 1 between treatments TC and LD. Note that the last temperature raising step from storage to experimental conditions in treatments TC and LD was 11 hours prior to switching to LD conditions, which roughly coincides with mean emergence phase in LD for the first 6 days.

6.2. Other supplemental Figures

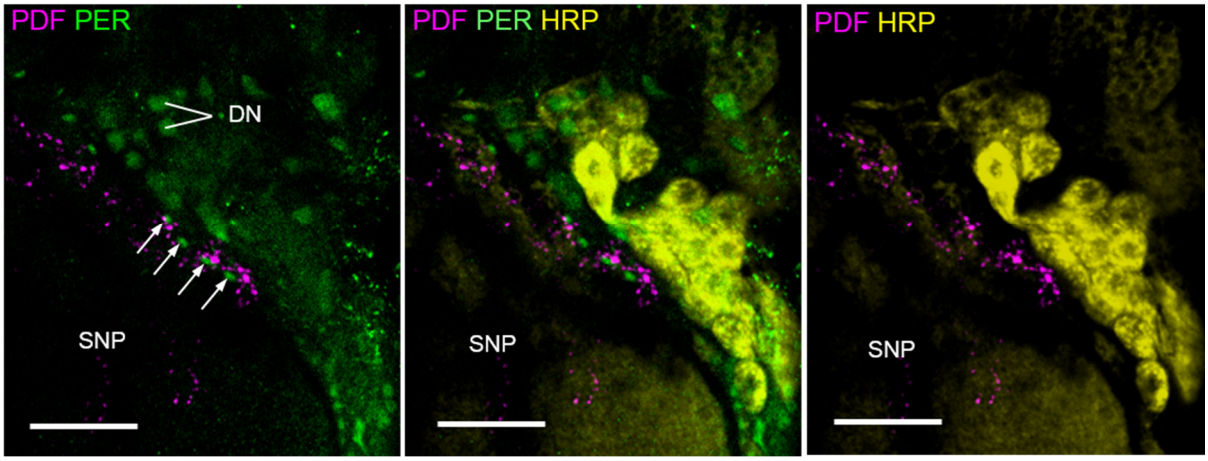

Figure S 3: PER-positive glia cells accompany the PDF fibers running into the superior neuropils.
Single confocal stack of a vibratome section stained with anti-PER (green), anti-PDH(PDF) (magenta) and anti-HRP (yellow) antibody. PER-positive glia cells (arrows) can be distinguished from PER-positive neurons by the lack of HRP. They are located in a row at the border of the superior neuropils (SNP) in close vicinity to PDF fibers. The PER-positive dorsal neurons (DN) are located dorsally from the PDF fibers which are running into the dorsal central brain and are occasionally contacted by PDF fibers crossing between the median and lateral calyces to the posterior side of the brain (not visible in this stack).

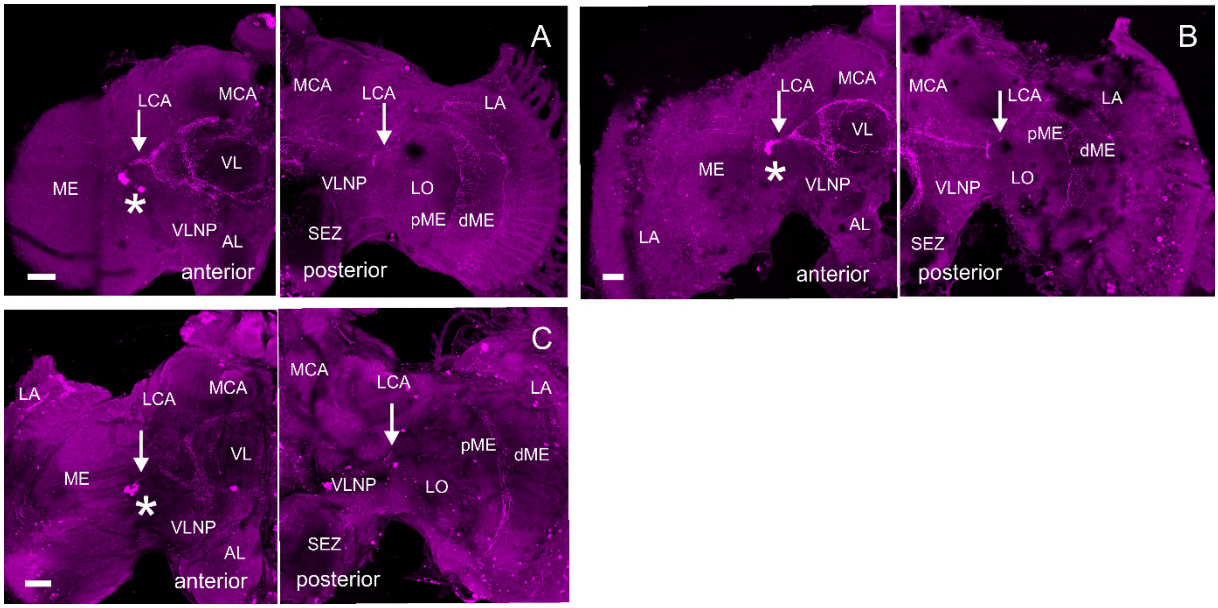

Figure S 4: PDF network in honey bees of pupal stages P3, P5, P7.
Frontal projection pictures of brains of pupal developmental stages P3 (**A**), P5 (**B**) and P7 (**C**). Laterally located PDF neuron somata (asterisks) project dorsally into a high density PDF network (ALO, anterio-lobular PDF hub) (white arrow) where neuronal arborizations intermingle. From here an extensive fiber network runs into the OL and the central brain. Fiber tracts grow densely during development. The anterior and posterior half of the whole mount preparations are shown in projection pictures separately. Arrowheads mark unspecific staining due to remains of pupal fat tissue. LCA and MCA: lateral and medial calyx of the mushroom bodies, VL: vertical lobe of the mushroom bodies, VLNP: ventro lateral neuropils, AL: antennal lobe, SEZ: subesophageal zone, LA: lamina, pME and dME: proximal and distal layer of the ME.

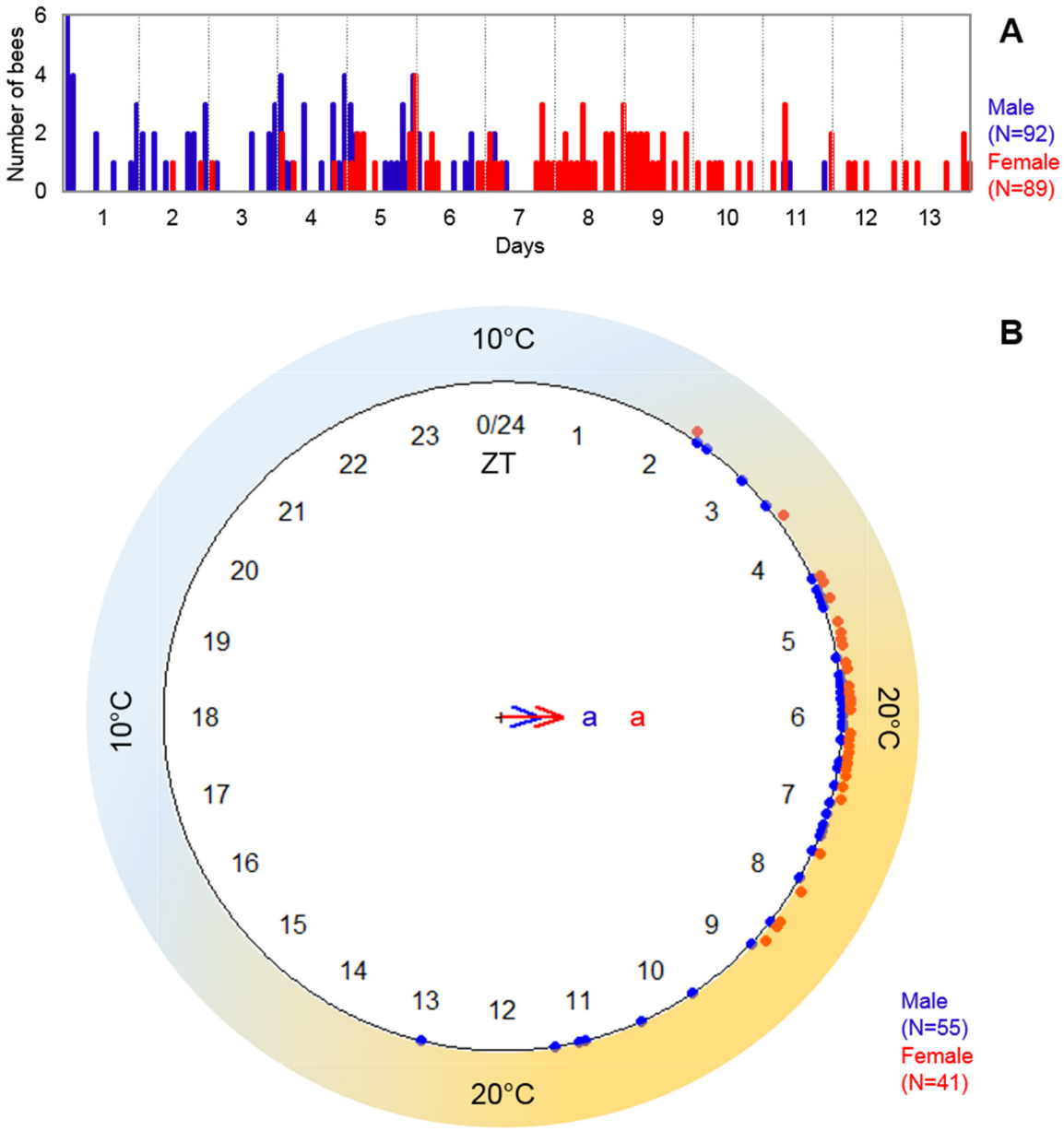

Figure S 5: Emergence of male and female *O. bicornis* in DD or a natural like temperature cycle. **A:** Histogram of emergence events of male and female bees (per 2h time bin) under DD conditions (20°C, 45% RH) after entrainment with temperature cycles (12h 20°C; 12h 10°C) for four days. **B:** Circular 24h plot of average day for emergence events of male and female bees (per 1 min time bin) under a natural like temperature conditions (hour ZT 0-6: temperature ramp from 10°C to 20°C; hour ZT 6-12: 20°C; hour ZT 12-18: temperature ramp from 20°C to 10°C; hour ZT 18-24: 10°C). The putative sex difference indicated by a tendency of male bees emerging earlier than females on the subjective day (**A**) was rebutted in the control experiment (**B**) with no difference in distribution of male and female emergence phases (Watson U^2 test: $p > 0.05$). The best-suited emergence temperature seems to be around 20°C for both sexes.

6.4. Submitted manuscript: Pigment-Dispersing Factor expressing neurons provide an infrastructure for conveying circadian information in the honey bee brain

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Abbreviated title: PDF is part of the bee clock
Number of pages: 49
Number of figures: 10
Number of tables: 0

Abstract
Pigment-Dispersing Factor (PDF) is an important neuropeptide of the circadian clock in many insects, but its role in bees is not clear. We combined high-resolution neuroanatomical characterizations, quantification of PDF levels over the day, and brain injections of synthetic PDF peptide to study role of PDF in the honey bee *Apis mellifera*. We show that PDF co-localizes with the clock protein Period (PER) in laterally located clock neurons and that the widespread arborisations of the PER/PDF neurons are in close vicinity to other PER cells (neurons and glia), suggesting that PDF modulates the activity of the latter. PDF immunostaining intensity oscillates in a diurnal and circadian manner in different parts of the bee brain, whereby synchrony of oscillations in different brain areas seems to depend on task or age. Finally, PDF injection into the area between optic lobes and the central brain at the end of the subjective day phase delayed circadian rhythms in locomotor activity. Altogether, these results are consistent with the hypothesis that PDF is an integral part of the honey bee clock that signals within the clock network and may transfer rhythmic signals to brain centres involved in activity control, time memory and sun-compass orientation.

Abbreviations:
AME: accessory medulla
AL: antennal lobe
ALO: anteriolobular PDF hub
AOT: anterior optic tract
AOTU: anterior optic tubercle
CBL: lower division of the central body
CBU: upper division of the central body
CX: central complex
DLN: dorsolateral neurons
dME: distal medulla
pME: proximal medulla
DN: dorsal neurons
ES: esophageal foramen
HRP: horseradish peroxidase
LA: lamina
LAL: lateral accessory lobe
LCA: lateral lobe of the calyx
LN: lateral neurons
LO: lobula
LOC: lateral ocelli
LVT: lobula valley tract
MAL: medial accessory lobe
MBDL: median bundle
MBU: medial bulbs
MCA: medial lobe of the calyx
ME: medulla
MOC: median ocellus
NO: noduli
OC: ocelli
PB: protocerebral bridge
PDF: pigment-dispersing factor
PED: peduncle
PER: period protein
POC: posterior optic commissure
POTU: posterior optic tubercle
RE: retina
SEZ: subesophageal zone
SNP: superior neuropils
VL: vertical lobe of the calyx
VLNP: ventrolateral neuropils

1. Introduction

The remarkable time memory ("Zeitgedächtnis") of foraging honey bees (*Apis mellifera*) was one of the first evidence for the functional significance of the endogenous clock in the brain [1,2]. It enables honey bees to synchronize flower visits with the daily floral rhythms maximizing pollen and nectar rewards [3]. Furthermore, in time-compensated sun-compass orientation, forager bees use the circadian clock to compensate for the movement of the sun across the sun over the course of the day [4,5]. The time-compensated sun-compass is also important for waggle dance communication in which honey bee workers refer to the sun position in the sun when recruiting nest mates to newly discovered floral patches or nesting cavities [6]. Plasticity in circadian rhythms is also link to the division of labour that is important for the social organization of the honey bee colony [7].

However, the neuroanatomical organization of the bee circadian network and its connections with brain centres that control memory and orientation, such as the mushroom bodies and the central complex are yet unknown. Neurons that express the neuropeptide "Pigment-Dispersing Factor" (PDF) play crucial roles in the clock network of many insects and, due to their global branching pattern in the insect brain, are suitable candidates to provide time-information to these centres. Such neurons have been initially described in cockroaches, crickets [8], flies [9–11] and later in a variety of other insects including bees [12–15]. In cockroaches, crickets and flies, the PDF neurons have dense arborisations in a small neuropil at the base of the medulla that is called accessory medulla (AME) and is regarded as the circadian pacemaker centre in these insects (Helfrich-Förster et al., 1998; Stengl and Arendt, 2016). In flies and honey bees, the PDF neurons express the clock protein Period (PER) and can therefore be regarded as bona fide clock neurons [16,17].

PDF neurons have been shown to be essential for circadian rhythms in several insects, including species in which co-expression of PER and PDF has not been reported so far [18–25]. In fruit flies, the intensity of PDF-immunostaining as well as the arborisation complexity of the PDF-terminals in the central brain was shown to oscillate during the day which was interpreted as circadian release of PDF [26–28]. Besides being an output factor of the fly circadian clock, PDF also functions as a key communication factor within the circadian network [29–31]. Consistent with this premise, most clock neurons express PDF receptors allowing them to respond to the PDF signal [32]. In cockroaches and crickets, injections of PDF into the optic lobes phase shifted rhythms in locomotor activity and in visual sensitivity which is consistent with similar circadian functions in these insects [33–36]. In honey bees *Pdf* mRNA levels oscillate, but cycling of the peptide has not yet been reported [14].

This study tests the hypothesis that PDF is a putative communicator in the neuronal circadian network in the honey bee brain and whether it has connections to brain centres controlling complex behaviour. To meet this goal, we performed a three-dimensional (3-D) characterization of PER and PDF neurons in the honey bee brain and studied PDF staining intensity in selected fibres as a function of time of day, or worker task. Finally, we injected synthetic honey bee PDF peptide into the honey bee brain to test whether it affects circadian rhythms in locomotor activity.

Material and Methods

1.1. Honey bee colonies

Honey bee colonies used for our studies were kept according to standard beekeeping practices at the Hebrew University of Jerusalem (Israel), the University of Regensburg (Germany) and the University of Würzburg (Germany). Each stock was composed of a mixture of subspecies typical to their region. If not stated otherwise, the queens of the colonies we studied mated naturally (with multiple drones). To obtain bees of known age we removed honeycombs with newly emerging bees and late stage pupae (identified by their relatively dark body pigmentation and purple to dark eyes [37,38]). The adult bees were removed and the brood comb was transferred in a lightproof cage into an incubator (33°±1° C, 55±5% RH) for 24 hours. After 24 hours, we collected newly emerging bees (0-24 h of age), marked them with a dot of coloured paint (Testor's Enamel) on the dorsal part of the thorax between the wings and introduced them back into their mother colony.

1.2. Detailed characterization of PER and PDF immunostaining

1.2.1. PDF immunostaining and 3-D reconstructions

For the 3-D description of PDF immunostaining we collected forager bees of unknown age from one colony at the Department of Zoology in Regensburg. Forager bees returning to the hive with pollen loads were collected at the entrances at the late afternoon during spring (~12 h day length) and were immediately dissected and processed for fluorescent immunocytochemistry. Whole brains were fixed overnight in Zamboni's fixative (4 % paraformaldehyde, 7.5 % saturated picric acid solution in 0.1 M PBS, pH 7.4), rinsed (3 x 10min) in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and 3 x 10min in PBS containing 0.5% Triton (PBST 0.5 %). They

were subsequently blocked in 5% normal goat serum in PBS overnight at 4°C. Polyclonal anti ß-PDH raised in rabbits against the crab ß-Pigment-Dispersing Hormone (PDH; Dircksen et al., 1987) was applied at 1:3000 (in PBST) for 5 days in order to guarantee that the antibody penetrated throughout the brain. This crab antibody stains reliably the PDF-positive neurons of insects, including honey bees [8,16,17]. After 4 days incubation with the secondary fluorescent antibody (Alexa Fluor 555, goat anti-rabbit, 1:200) the brains were incubated over night with Lucifer Yellow (1:12,800) to stain the neuropils. The brains were washed with PBS (5x10min), dehydrated in ethanol (30, 50, 70, 90, 95, 3 x 100 %; each step 20 min), cleared in methyl benzoate (50:50 ethanol: methyl benzoate for 20 min, 100% methyl benzoate overnight) and embedded in Permount (Fisher Scientific, Schwerte, Germany) between two coverslips separated from each other with spacers to prevent squeezing of the brains.

Confocal images were obtained using a Leica TCS SPE confocal microscope with a 10x air objective (numerical aperture: 0.3). The whole-mount brains were scanned at a resolution of 1024 x 1024 in the xy direction and a step size of 2.39 µm in the axial direction. Because of the thickness of the brains, each brain was scanned in anterior and posterior image stacks, which were later aligned in z-direction in the Amira® software (version 4.1.1) by using the landmark tool.

One brain showing the best PDF staining (out of five) was reconstructed with Amira®. Neuropils were reconstructed in the segmentation editor and, after subsequent resampling, a smoothed surface 3-D model was generated. PDF fibres were reconstructed using the "Neuron Tracer Plugin". Here we added a high number of tracing points along the fibres and smoothed reconstructed structures in order to avoid calculation errors in dense fibre tracts. The varicosities of the PDF fibres are generally not preserved in the neural network reconstructions. To demonstrate the varicosities, we show original confocal images of certain brain areas in addition to the reconstructions.

1.2.2. PER and PDF double staining

The PER and PDF double staining experiments were performed in Würzburg. We generated 14 preparations of whole-mount brains and vibratome sections based on honey bees from two colonies kept at the Department of Animal Ecology and Tropical Biology. Pollen forager bees were collected at the hive entrances at the late afternoon during spring and summer (LD was approximately 11:13 h in the whole-mounts experiments (sunset = 18:00), and approximately 16.5:7.5 h for the vibratome experiments (sunset = 21:30) and placed inside an incubator until sampling (20 ± 0.5°C, 60 ± 10 % humidity, approximately 200 lux during light phase; light was switched off at natural sunset). They were sacrificed during their first night in the incubator 2 hours before the time of sunrise, which mimics the time of high PER, respectively [13,17] and immediately processed for immunocytochemistry. Since both antibodies, anti-*am*PER and anti-βPDH, were raised in rabbit [17] we performed two separate staining procedures with an additional fixation in Zamboni's fixative in between (for details see [17]). This additional fixation of the brain samples in Zamboni's fixative is assumed to denature remaining free binding sites of the anti-PER antibody which successfully prevented that the second secondary antibody recognized PER and allowed us to unequivocally distinguish between PER and PDF staining. The incubation in anti-PER solution was 7 days for whole-mounts and 2 – 4 days for brain sections, both at 4 °C (anti-PER was diluted 1:1000 in PBST for whole-mounts and 60 µm vibratome sections; in 150 µm vibratome sections the antibody was preabsorbed on per^0 *D. melanogaster* mutants to reduce non-specific staining, and was diluted 1:100). The incubation in anti ß-PDH was 7 days for whole-mounts and 4 days for sections (dilution 1:3000). One representative detail scan of a whole-mount brain showing PER- and PDF staining in the lateral brain was used to reconstruct the projections of the PDF neurites in relation to the PER-positive nuclei with Amira® software (version 6.1.1) using the "Filament tracer" tool.

1.2.3. Additional HRP and DAPI staining

Some of the PER and PDF immunostained vibratome sections were additionally labelled with a fluorochrome-coupled anti-HRP (Horseradish Peroxidase) antibody which immunostains the neuropils and the somata of neurons. The HRP antibody recognizes a carbohydrate residue of the neuron-specific cell surface protein Nervana [39]. Therefore, HRP labels the surface of all neurons (cell bodies and neurites) but not glial cells, and leaves the nuclei unlabelled [40]. For the HRP staining, we incubated the tissue with Cy3-AffiniPure Goat Anti-HRP (Catalog No. 123-165-021, Jackson ImmunoResearch, West Grove PA, USA) diluted at 1:300 in PBST 0.5% (with 5%NGS and 0.02% NaN_3) for 48h at room temperature and washed 5 times in PBST (0.5% Triton). In the 5th washing step we added DAPI (4-6-diamidino-2-phenylindole dihydrochloride hydrate; Sigma D9542; Eugene, Oregon, USA; 1 mg/ml washing solution) to allow for nuclear counterstaining, and washed 3 times in PBS (details see [17]). Afterwards the sections were mounted in Vectashield.

1.2.4. Nomenclature

We followed the naming conventions for neuropils suggested by Ito et al. [41] wherever possible. The naming of the clock neurons is adapted to *Drosophila melanogaster* and our recent definitions for *Apis mellifera* [17]. For simplicity, we will refer to the neurons that are stained with the PER antibody and/or the PDH (PDF) antibody, "PER-positive" and "PDF-positive" neurons or simply "PER and PDF neurons", respectively. Similarly, we will refer to fibres arising from the PDF neurons, as "PDF fibres".

1.3. Diurnal and circadian variation in PDF staining

The first two experimental trials were performed with bees from the Hebrew University of Jerusalem in October (11.6 h day length). The queens of the colonies used for these experiments were inseminated with the semen of a single drone (different for each colony) in order to reduce genetic variation among sister workers (honey bee queens naturally mate with 10-20 males). Foragers and nurses were collected either every six hours (ZT4, ZT10, ZT16, and ZT22, whereby ZT0 is defined as sunrise) or every four hours (ZT2, ZT6, ZT10, ZT14, ZT18, and ZT22) directly from a hive, in which the foragers could fly outside and were entrained by the natural light-dark cycle. The forager bees were identified based on their conspicuous pollen loads and were paint marked during the day to enable identification at times when not foraging (e.g., during the night). Nurses were identified worker bees with their head inside a brood containing honeycomb cell. Brains were quickly dissected, fixed and shipped in the fixative solution to Regensburg for immunofluorescent treatment (see below).

Experiments three and four were entirely performed in Regensburg during spring (day length 12.5h) and included only foragers (identified as above). Since there was no possibility to access the hive at night, the foragers were collected directly at the hive entrance of one bee colony in the evening just before sunset (~18:00) and immediately transferred to an incubator with a 12:12 LD regime, in which the light was switched-off at natural sunset (28 ± 0.5°C, 80 ± 2 % humidity). In experiment three, the bees were entrained for 3 to 4 days and then dissected at 6 different time points (ZT2, ZT6, ZT10, ZT14, ZT18 and ZT22), while in the fourth experimental trial bees were kept in constant darkness (DD) for 3 days and then dissected on the fourth day every 4 hours.

1.3.1. Anti-ß-PDH staining for samples collected around the day

For quantitatively analysing the ß-PDH staining intensity, all brains within one experiment were processed together and treated exactly in the same way (keeping times of fixation, rinsing, and antibody incubation constant). The brains were fixed in Zamboni's fixative for 4 days, rinsed and embedded in gelatine-albumin (12g gelatine (Sigma, 300 bloom), 75g ovalbumin (Sigma, A 5253, albumin chicken egg grade III) in 250 ml distilled water). The gelatine-albumin blocks containing the brains were fixed again in 4% PFA at 4°C overnight and then stored in PBS. The blocks were cut with a vibratome (Leica, VT1000 S) in 100 µm thick frontal sections. Fluorescent anti-ß-PDH staining was then performed on the floating vibratome sections as stated in "PDF immunostaining" but with shorter antibody incubation times (anti-ß-PDH 1:3000 for 48h, ALEXA Fluor 488 goat anti rabbit 1:200 overnight).

1.3.2. Analysis of PDF staining intensity

PDF staining intensity of the first two experiments (4 and 6 time points under naturally synchronized conditions; bees from Jerusalem) was quantified as described in Park et al. [26]. Briefly, the area of interest was chosen for each brain (see Fig. 9). The average intensity in grey values of 10 points (pixels) in the background close to stained structures was subtracted from the average intensity of the 10 brightest points in PDF stained structures. In experiments three and four the 10 brightest points were close to saturation (255 pixels) at the maxima of PDF-cycling. Therefore, we quantified staining intensity by using the protocol described in Hermann-Luibl et al. [28]. Briefly, 10 confocal stacks containing the relevant PDF-structure were merged and background was set to zero by adjusting "intensity" of the image in Corel Photopaint (version X6, 64bit). All labelling outside the relevant structure was manually removed and the pixel intensity of the entire image determined in ImageJ. If not stated otherwise, staining intensity was determined in the relevant structure of both brain hemispheres and an average staining intensity calculated for each brain.

Statistics. First, we tested whether the data were normally distributed by the Kolmogorow Smirnov Test. If this was the case, we applied a one-way ANOVA to reveal significant influences of the time of day on PDF staining intensity. If this was not true, a non-parametric Kruskal-Wallis test was applied to the data.

1.4. The influence of PDF injections into the brain on circadian rhythms in locomotor activity.

These experiments were performed at the Hebrew University of Jerusalem. We used foragers (identified by pollen loads on their hind legs), or forager-age (21-22 days old) bees which typically have strong circadian rhythms (reviewed in [7,42]). The bees were collected from two colonies: one headed by a naturally mated queen, and one headed by a queen instrumentally inseminated with semen of a single drone (which reduces genetic variation within the colony). We first performed a set of preliminary

experiments in which we confirmed that cold anaesthesia and the injection procedure in our protocol do not affect bee survival and circadian rhythmicity (data not shown).

1.4.1. Monitoring of locomotor activity and determining the effects of PDF on circadian rhythmicity

We collected 11-day-old bees into wooden cages and synchronized the bees for 4 days in a LD 12:12 regime. During the dark phase we transferred them in a light sealed box into an environmental chamber (28 °C, 60% RH) with constant dim red light (>640 nm, peak at around 680). Each bee was placed individually in a monitoring cage made of a modified Petri dish (9 cm diameter, 1.5 cm height), provisioned with 50 % (w/w) sugar water. We monitored locomotor activity with the ClockLab data acquisition system (Actimetrics CO., U.S.A.) with 4 light-sensitive black and white Panasonic WV-BP334, 0.08 lux CCD cameras (each camera recorded activity from 30 cages), and a high-quality monochrome image acquisition board (IMAQ 1409, National Instruments Co., U.S.A.) [38,43]. Data were collected continuously at a frequency of 1 Hz. On the fifth day after transfer into the locomotor activity monitoring system we injected the bees either with saline or with PDF dissolved in saline using two different methods (see below). An additional control group included bees that were similarly handled and anaesthetized but were not injected. All manipulations were done 8-11 hours after the onset of the bees' locomotor activity. This corresponds to circadian time (CT) 8-11 (= CT8-11) and is around the end of their subjective day, a time at which we expect prominent phase delays according to studies performed in cockroaches [33,34]. The injected bees were returned to the monitoring system and their activity was recorded for additional 4 days in constant dim red light. To calculate putative phase shifts induced by the treatment, we fitted regression lines to the onset of activity during the four days before, and the four days after the day of injection. The difference between the interpolations of the two regressions lines on the day following the day of injection was used for estimating the phase shift. We also compared the days before and after injection in terms of: (1) the free-running period (ΔFRP) and (2) the strength of circadian rhythms (ΔPower). The effect of the treatment was analysed using the non-parametric Mann-Whitney test (SPSS Inc.).

1.4.2. Synthetic PDF peptide.

The amidated *Apis mellifera* Pigment-Dispersing Factor (*Am*PDF) was synthesized by BioSight Ltd. Peptide Technologies (product No. 07-01-0013-1) based on the PDF orthologue sequence predicted in the honey bee genome (NSELINSLLGLPKNMNNA - Amide; [44]). The peptide was dissolved in ultra-pure water to reach a concentration of 2mM PDF, divided to 100µl aliquots and frozen in -80°C until use. We performed Mass Spectrometry and HPLC tests to determine the purity and estimate the amount of PDF peptide in the solution before using the peptide. The amount of injected PDF was corrected based on these estimations. The peptide solution was mixed with honey bee saline to reach a final concentration of 0.001mM PDF, 0.01mM PDF and 0.1mM PDF. Control bees were injected with saline alone that was prepared by a standard protocol [45] and kept at -4°C before use.

1.4.3. PDF and saline injections

We used two different protocols for the injections. The first protocol was based on similar injection studies with cockroaches [33] and crickets [35] and requires the removal of parts of the head capsule cuticle. In the second newly developed protocol, we punctured a small opening in the head cuticle without removing it. In both cases, we injected only into the right optic lobe, whereas the left lobe was left intact. Furthermore, in both cases the bees were anesthetized by chilling them down to a temperature of ~1°C . The whole procedure was performed under a dissecting microscope equipped with red light illumination (optic fibre system with red light filters; >640 nm, peak at around 680). Care was taken not to expose the bees to white light.

The injections themselves were performed using an electric nano-litric injector (Nanoject II, Drummond, cat. No. 3-000-205) loaded with a glass electrode (Drummond 3.5", cat. No. 3-00-203-G/X) pulled with a micropipette puller P-97 (Sutter Instruments Co.). The electrode was first filled with heavy mineral oil (Sigma) that is not compressible, enabling a more accurate volume injection and then loaded with ~50µl of injection solution. The micro-injector was directed with a mechanical micromanipulator (Right 3-000-024-R) to the desired position.

1.4.3.1. Injections after cutting a window into the head capsule

The bee was fixed with a strip of commercial play-doh that was placed over the head capsule on a custom-made ice-chilled injection platform. We cut a small window in the head capsule using a delicate scalpel and fine tweezers. First, two incisions were cut through the cuticle; the first incision starting between the antennae base and ending between the ocelli and the tip of the compound eye, the second incision was made along the frontal side of the compound eye until merging with the first incision at the dorsal tip of the head. Then, the cuticle piece, which was still connected to the bee head at its base, was flapped down and held in its position with a stapling pin. The brain and surrounding tissues was

now visible. The electrode was directed to the middle of the vertical axis passing through the lobula, which was easy to locate. Once in position, the electrode was inserted, penetrating the brain tissue to a 20 µm depth from its external surface (as measured by the turning of the micromanipulators' small screw). 2.3 nl of the solution was injected into the tissue and the electrode was retracted from the brain. After the injection, we reattached the piece of cuticle by placing hot commercial bee wax (obtained from Yad Mordechai apiary, Israel) over the incision. The entire procedure, from anaesthesia to the sealing of the cuticle, lasted between 7-15 min.

1.4.3.2. Stereotactic injections through the head capsule cuticle without cutting a window.
For this protocol, we designed and built a new custom-made aluminium fixation mould, which enabled uniform fixation of the bee in such a way that the anterior surface of the head was perpendicular to the nano-injector (Fig. S1a-c). The fixation mould was designed with a wide base to allow efficient and uniform chilling (~1°C) of the bee without wetting (Fig. 1a, c). The bee was fixed on the mould by inserting its neck trough a narrow cleft (Fig. S1c) and harnessing her with soft play-doh strips on the head and back. Injection site was determined with the help of a gridded stereomicroscope lens (using X40 magnification). The vertical scale of the grid was oriented in such a way that it reached from median ocellus until the area in between the origin of the two antenna (Fig. S1d). The ocellus was placed exactly at the vertical scale mark 25, and the injection was done at scale mark 25 on the left arm of the grid (Fig. S1d). The cuticle was pierced with the tip of the electrode and the latter was inserted 1.2 mm from the cuticle surface into the bee brain tissue. The electrode was then retracted by 0.2 mm and two consecutive injections of 2.3 nl were made (total = 4.6 nl). Next, the electrode was fully retracted out of the bee's head and the bee was immediately placed in its cage for recuperation. The entire stereotactic injection procedure took only 2.5 min, which is significantly shorter than the first procedure.

1.4.4. Verifying the site of injection
For the first protocol, in which injection site was visually determined based on brain morphology, we verified the anatomical location by using the same injection protocol but with a fluorescent dye (DiI, Molecular probes, D-3911 diluted 1:1000 in EtOH). Following the injection, we fixed the brains and performed fluorescent PDF immunocytochemistry as described above.

For the second protocol, we estimated the anatomical site of injection retroactively. We mixed the solutions with 2% blue dye (Chicago Sky Blue powder 6B, SN. C8679; Sigma LTD) to reveal the injection site. This dye binds covalently to the cell membrane for up to 2 weeks. It is widely used in neurobiology and does not seem to affect cell function significantly [46].

At the end of the experiment (five days post injection), we sacrificed the bee and opened the head capsule to expose the brain (Fig. S1d). We photographed the brain of each bee with a digital camera (Nikon) under X50 magnification Then, each injection mark on the photograph was digitally measured and given X and Y coordinates. The distance between the two saddles (i.e., between the optic lobe and central brain) was measured and used as a normalization factor allowing precise site localization for bees differing in brain size. All the normalized coordinates were combined and displayed on a reference figure of a brain stained with anti PDF antibody, using a specific algorithm (MATLAB). Then, the presented data was combined with an actual anatomical image that was dyed against PDF (Adobe Illustrator CS4) (figure 10c,d). This imaging enabled us to look at the effect of the injections (phase shift) as a function of the estimated injection site relative to PDF-positive neurons.

2. Results

2.1. The PDF neurons comprise a subgroup of the PER-positive neurons that arborize throughout the brain.

The clock protein PER is expressed in four major neuronal clusters in the lateral and dorsal brain of the honey bee (Fig. 1a; [17]). PDF co-localizes with PER in the Lateral Neurons 2 (LN_2) cluster that in foragers consists on average of 15.2 (± 0.4) neurons with rather large somata (Fig. 1a). The PDF fibres emanating from the LN_2 cluster invade most parts of the brain, including the optic lobes, the ocellar tract and the antennal lobes, and with a particularly dense branching pattern in the protocerebrum (Fig. 1b, c; see also [13] and [14]). Due to the massive overlap of the PDF fibres it was impossible to unambiguously assign projections to individual neurons. Nevertheless, using detailed 3-D reconstruction we were able to unravel neuroanatomical details that have not been described before, neither in sections [13] nor in whole-mount labelling [14]. Below we describe novel features in the projection pattern of the entire population of PDF neurons with emphasis on fibres passing close to PER-positive cells, adjacent to the mushroom bodies or to the central/lateral complex, and in brain regions in which we later recorded PDF immunostaining intensity. A more detailed description of the PDF projections can be found in [47] and a movie demonstrates the PDF fibres in 3-D (Suppl. Mat).

2.2. Projection pattern of the PDF neurons

2.2.1. Fibres of the PDF neurons form a high-density PDF-network in front of the lobula.

The somata of the bee PDF neurons were located proximally of the medulla and are heterogeneous in size. One PDF neuron with rather big soma was located most anteriorly (shown in red in all 3-D reconstructions). The remaining somata could hardly be classified into size categories; they varied in width from 7 to 21 µm and in length from 10 to 29 µm, as already reported by Bloch et al. [13] and Fuchikawa et al. [17].

On their way towards the central brain, all PDF neurons project into a high-density network located in front of the lobula (red arrows in Figs. 2-4), which we call anterolobular PDF hub (ALO), and not into a small neuropil at the base of the medulla, the AME, as in other insects. The AME in our preparations could not be clearly demarcated by immunostaining for PDF. To better describe the neuroanatomy of this brain region, we further used HRP and DAPI counterstaining which mark neurons and cell nuclei, respectively. With this approach, we identified a small neuropil structure at the base of the medulla, which is reminiscent of the AME of other insects. This neuropil was free of nuclei and innervated by PDF fibres (Fig. 2), which continued into the serpentine layer of the medulla (arrowhead in Fig. 2; blue fibres in Figs. 4, 5) and to a minor degree into the most distal layer of the medulla and into the lamina (double arrow head in Fig. 2; red fibres in Figs. 4, 5). Nevertheless, the innervation of the small neuropil structure by PDF fibres was not very prominent, but of similar density as the innervation of the medulla. It was clearly less dense than the ALO (red arrow in Figs. 1-3).

2.2.2. Vicinity of PDF-positive fibres and the PER-positive LN_1 and DLN neurons.

PDF fibres from ALO pass through the PER-positive LN_1 cluster and seem to touch many of these cells (Figs. 2, 3a,b). Subsequently, the PDF-positive fibres run towards the central brain along the anterior surface of the lobula. At the proximal anterior rim of the lobula several PDF fibres leave the network and project to the superior and, to a minor degree, also to the ventrolateral neuropils of the anterior protocerebrum (SNP and VLNP; Fig. 3; green double arrow head in Fig. 4a). On their way, the PDF-positive fibres running to the SNP pass by the dorso-medial part of PER-positive DLNs cluster, in which they appear to be in close proximity to a subset of these neurons (white arrows in Fig. 3a,c).

2.2.3. The lobula valley tract.

The majority of PDF fibres of the ALO do not leave the surface of the lobula but turn posterior and join a compact tract that continues to run along the surface of the lobula until it reaches the lobula's ventral posterior rim (white fibre tract in Fig. 4). This conspicuous tract corresponds most likely to the lobula valley tract (LVT) that was described in detail for the cockroach and that serves as a kind of "highway" connecting clock centres in the AME with the protocerebrum as well as the ipsi- and contralateral AME [48]. This tract seems to play a similar role in the honey bee because most if not all PDF fibres seem to canalize in the LVT. They seem to enter the LVT from different brain regions and to leave it at various places. For example, on the way of the LVT to the posterior lobula many PDF fibres leave or enter it (green fibres in Fig. 4). The latter run on the posterior surface of the lobula and connect the lobula with the serpentine layer of the medulla (the green fibres meet the blue fibres in Fig. 4d). We do not know whether these fibres leave the serpentine layer of the medulla and run over the posterior surface of the lobula into the LVT, or *vice versa*. The same is true for all other fibres that originate from the LVT: they may enter or leave the LVT. After having reached the posterior rim of the lobula the honey bee LVT makes a characteristic loop and turns back to the anterior lobula (Fig. 4a,b). Then it "dissolves" in many small fibres innervating the protocerebrum (green in Fig. 4b).

Just before the LVT turns back anteriorly, several fibres leave it and enter the posterior optic commissure (POC), or *vice versa*, fibres coming from the contralateral hemisphere leave the POC (orange fibres in Fig. 4c,d) and enter the LVT close to its loop. Other PDF-positive fibres leave or enter the LVT loop from fibre bundles running to, or coming from, ventral parts of the protocerebrum (Fig. 4e). The "loop" of the LVT can be easily discerned in posterior vibratome or confocal sections and therefore we later quantified PDF staining intensity in it (see rectangle #4 in Fig. 8b). Most interestingly, PER-positive glia cells are aligned at the posterior rim of the lobula and some of them seem to be in close proximity of the PDF-positive fibres in this place (Fig. 4f).

2.2.4. PDF-positive fibres extending into the lamina

The fibres running in the distal layer of the medulla (fibres marked by a double arrowhead in Fig. 2; red fibres in Fig. 4) do not remain in the medulla, but extend into the lamina (Fig. 5). Most of them leave the medulla dorsally (in its dorsal rim area) as can be seen best in the left optic lobe shown in Figure 5a. This optic lobe is an exceptional case, in which PDF-positive fibres running in the distal layer of the medulla are only present at the dorsal and ventral rim of the medulla. Usually, the PDF fibres form in addition a loose fan of distally projecting PDF fibres that extends over the entire surface of the medulla

(right optic lobe shown in Fig. 5b). This loose fan innervates mainly the proximal lamina, leaving the distal lamina devoid of PDF. Only in the most dorsal region (dorsal rim region of the lamina) some PDF fibres extend more distally (Fig. 5c). This is also the region, in which the PDF-fibre network between the medulla and lamina is rather dense and many PER-positive glia cells are located (Fig. 5c). It is difficult to judge whether the PDF fibres have direct contact to the PER-positive glia cells, but since they are very close to each other, this appears likely. A similar close vicinity between PDF fibres and PER-positive glia cells exists in the more ventral part between medulla and lamina (Fig. 5d), as well as between lobula and medulla (Fig. 5e), although in the latter area, the PDF network is not very dense.

2.2.5. PDF-positive fibres in the proto- and deuto- and tritocerebrum.

Most of the PDF fibres entering the protocerebrum in the midbrain form a dense network at its surface (green fibres in Fig. 6a). This network surrounds the mushroom bodies, but does not invade them (see also Fig. 7). A specifically dense PDF fibre network is present in the dorsal protocerebrum between the vertical lobes and the calyces of the mushroom bodies. This fibre network extends extremely close to the calyces of the mushroom bodies. We later determined the intensity of PDF staining in the main fibre bundle below the calyces that continues into the median bundle (see rectangle #2 in Fig. 9). Between the lateral and medial calyces, two individual PDF fibres leave this main bundle at the level of the PER-positive dorsal neurons (DN) (white arrowhead in Fig. 6a) and cross between the lateral and medial calyx towards the posterior protocerebrum (white arrow head in Fig. 7). In the anterior area between the lateral and medial calyx, the PDF fibres come close to the DN, (Fig 6c) and particularly to PER-positive glia cells that are aligned in a row between the dorsal protocerebrum and the calyces (white arrows in Fig. 6b,c; Fig. S2).

Medially, some fibres running between the calyces and the vertical lobes of the mushroom bodies extend into the median bundle (Fig. 6d). Others form an anterior dorsal commissure that projects to the contralateral brain hemisphere (Fig. 1, commissure 1, Fig. 6d, double arrowhead; see also below). According to its location this commissure might be homologous to the anterior optic commissure described in the cockroach [48]. In addition, we found a close relationship between PDF-positive fibres and PER-positive glia cells in the median bundle (Fig. 6d). Some fibres of the median bundle continue ventrally and invade the deutocerebrum, i. e. the dorsal part of the antennal lobes from posterior (white fibres in Fig. 6a). The deutocerebral PDF fibres are again loosely accompanied by PER-positive glia cells and arborize between the dorsal glomeruli of the antennal lobes (Fig. 6d). They do not seem to enter the glomeruli. Furthermore, in the ventral protocerebrum, just above the deutocerebrum, PDF fibres are close to PER-positive glia cells that are aligned in a row (white arrow in Fig. 6d). PDF fibres do also invade the tritocerebrum: posterior to the antennal lobes, they surround the oesophageal foramen, again closely accompanied by PER-positive glia cells (Fig. S3).

The anterior optic tubercles (AOTU; Fig. 6b) are surrounded by a dense PDF fibre network, but fibres do not enter them (Fig. S4). The AOTU of each brain hemisphere receives information from the compound eye via fibres from the lobula and medulla running in the anterior optic tract (AOT). Most interestingly, the AOT does also not contain any PDF-positive neurites (see HRP/PDF staining in Fig. 2). Thus, a major light input pathway to the protocerebrum is free of PDF fibres. Nevertheless, we found PDF in a second light input pathway to the protocerebrum, the posterior optic tubercles (POTU; see below). Ventrally of the vertical mushroom body lobes (VL), PDF-positive neurites extend towards the median brain and form a second anterior commissure (Fig. 6c,d; Fig. 1, commissure 7).

2.2.6. PDF-positive fibres in the middle of the protocerebrum surround the mushroom bodies and the central complex

The PDF fibres in the medial brain stem partly from the PDF network on the surface of the anterior brain shown in Figure 6 and partly from PDF fibres that leave the LVT on its way to the posterior rim of the lobula and enter the medial brain (see green arrowhead in Fig. 4a). Some of these fibres surround the vertical lobe and the peduncle of the mushroom body in a ring-like fashion (Fig. S5). Others form a conspicuous trapezoid network made of three triangles (two side ones with their tip up, and one with an opposite orientation connecting the two) behind the medial lobes of the mushroom bodies (Fig. 7a,b) and around the central complex (Fig. 7c-f). Part of this three-triangle network are two double commissures (Fig. 7c, d), which arborize broadly anterior and posterior of the dorsal and ventral edges of the central complex, respectively (Fig.1, commissures 2, 3 and 5, 6). The dorsal double commissure (Fig. 1, commissure 2, 3) runs anterior and posterior of the upper unit of the central body (Fig. 7d). The ventral double commissure (Fig. 1, commissure 5, 6) is part of the base side of the three-triangle network (Fig. 7b,c).

None of the PDF- fibres in the three-triangle network enter the central complex, but the fibres in its ventral part have a close relation to the lateral complex, neuropils that are associated with the central complex [49]. The lateral complex consists of the medial and lateral accessory lobes (MAL and LAL) and the medial and lateral bulbs (MBU and LBU)[50]. Many PDF fibres running in the ventral double-

commissure of the triangle network leave it in the middle and invade the medial accessory lobe (MAL), which is located directly underneath the lower unit of the central body (CBL) and the MBU (Fig. 7e). Anteriorly, these fibres appear to touch the median bulbs of the lateral complex (MBU, white arrow in Fig. 7e) and, posteriorly, they seem to extend to the noduli (NO; arrow in Fig. 7f), although they do not invade them. Furthermore, the lateral sides of the two triangles appear associated with the LAL (Fig. 7f). Throughout the three-triangle network PER-positive glia cells accompany the PDF-positive fibres in this area (Fig. S3).

Posterior of the two double commissures runs the POC, which connects the PDF neurons of the two hemispheres (orange fibres in Figs. 4, 5, 7). Several PDF fibres leave the MAL and the POC, project dorsally and invade the ocelli (Fig. 7g,h; Fig S6).

2.2.7. PDF-positive fibres in the posterior optic tubercles (POTU)

The POTUs are small neuropils located in the posterior brain, adjacent to the protocerebral bridge and the POC [8,51]. In locusts they are part of a potential second polarization vision pathway that run in the POC and arborize in the AME [52]. In cockroaches and locusts they are invaded by PDF fibres arborizing in the AME and leaving the POC [8,48]. In the honey bee, the POTUs are less well characterized, but we found similar fibres that leave the POC and arborize in these small neuropils (Fig. 7g). Furthermore, some PDF fibres leaving the POC project in the direct vicinity of the protocerebral bridge (Fig. 7h).

2.3. Temporal variation in PDF immunostaining intensity

If PDF is involved in transmitting time-of-day information, one would expect it to be rhythmically released into target brain areas. We tested this hypothesis by measuring PDF immunostaining intensity around the day in the following regions of interest: (1) the PDF neuron somata (Fig. 8, rectangle #1), (2) the area ventrally to the calyces of the mushroom bodies (Fig. 8, rectangle #2), (3) the median bundle (Fig. 8, rectangle #3), (4) the LVT at its most posterior location (Fig. 8, rectangle #4) and (5) the posterior rim of the serpentine layer in the medulla (Fig. 8 rectangle #5).

2.3.1. PDF oscillations in nurses and foragers under entrained conditions

The first two experiments compared PDF immunostaining intensity for forager and nurse bees from two different source colonies in Israel. We found time-dependent changes in PDF-staining intensity in both foragers and nurses: PDF-staining intensity was lowest at the beginning of the day and highest at the end of the day/ beginning of the night. In the first experiment that included 4 time points over a single day, there was a significant influence of time of day on PDF immunostaining intensity in all the focal arborisation areas ($p<0.05$; Fig. 8). Furthermore, we revealed a significant PDF cycling in the cell bodies of foragers and a significant oscillation in the number of immunostained cell bodies in nurses (Fig. 8). An overall very similar temporal pattern was obtained in the second experiment in which bees were sampled over 6 time points per day. However, in this experiment only the variation for the LVT fibres of foragers and the fibres ventrally of the calyces of nurse bees were statistically significant at the $p<0.05$ level. It should be noted that this experiment had lower statistical power because sample size was only 3-4 brains per time point (Fig. S7).

Taken together, these two experiments point to a possible difference between the oscillations in the PDF network of nurses and foragers: in nurses, the oscillations in the five focal areas that we measure showed high synchrony (correlation coefficient (r) between staining intensity in the PDF cell bodies and the different fibres was between 0.854 and 0.992 in the first experiment and between 0.871 and 0.932 in the second experiment). All the measured PDF-fibres show a sharp drop in staining intensity at the early morning (Fig. 8; Fig. S7). In foragers this drop appeared more gradual and overall PDF cycling was less synchronous among the focal areas ($0.027 < r < 0{,}653$ in the first experiment and $0.208 < r < 0{,}449$ in the second experiment) as compared to nurses (Fig. 8; Fig. S7).

2.3.2. PDF oscillations in foragers under constant dark conditions

This experiment was performed with bees collected in Germany. We quantified PDF staining intensity only in the fibres between the vertical lobes and calyces, in the LVT and in the serpentine layer of the medulla (#2, #4, and #5 in Fig. 8, respectively). We first repeated the 6-time-point experiment in LD 12:12 (this time under artificial light in an incubator) to compare PDF levels over the day in the bees from Germany and Israel. Although PDF immunostaining intensity in the fibres in this experiment was higher than in the two experiments with bees shipped from Israel, the temporal patterns were very similar: PDF staining in all areas of interest was higher during the night than during the day with a clear minimum occurring at the beginning of the day (ZT2; Fig. 9). An ANOVA revealed significant time-dependent differences in all focal PDF fibres areas ($p<0.05$). Furthermore, the variation over the day in the different PDF fibres were again slightly out of phase as revealed by rather low correlation coefficients between the three areas of interest ($0.10 < r < 0{,}38$).

Next, we assessed PDF levels in foragers sampled under constant darkness and temperature over a single day, thus, representing genuine endogenous rhythms. We sampled foragers and kept them for 3 days in a constantly dark incubator. We processed samples of bees collected every four hours for PDF-immunocytochemistry on the fourth day in DD. The results presented in the lower panel of Figure 9 show PDF-immunostaining intensity throughout one cycle in circadian time (CT). To estimate circadian time, we assumed that the bees are free-running with a period of 23.5 h (conferring to the free-running periods measured previously for bees from a comparable German apiary [53]) and accordingly treated each day as lasting 23.5 rather than 24 hours (i.e., CT 24 is actually 23.5 hrs after CT0). Indeed, visual inspection of the curves reveals oscillations in PDF staining intensity with the expected phase: in all three areas of interests, PDF staining intensity was higher during the subjective night than during the subjective day (Fig. 9). However, the time effect in an ANOVA was statistically significant only for the serpentine layer of the medulla (blue line in fig 9; p=0.01). The lack of significant differences in the two other areas of interest may stem from the rather high variability among individual bees that differ in their free-running period.

2.4. The influence of PDF injections into the brain on circadian rhythms in locomotor activity

To further test the hypothesis that PDF signalling is important for circadian rhythms in honey bees, we tested the influence of PDF injection on circadian rhythms in locomotor activity. We used two different protocols to inject PDF at CT 8 to CT11 into one side of the brain, laterally to the PDF-positive somata.

2.4.1. Injections after cutting a window in the head capsule cuticle

In the first sets of experiments, we opened a window in the head capsule cuticle, and injected PDF or saline into one optic lobe, laterally to the PDF-positive somata. A similar injection of a fluorescent dye (Fig. 11a) confirmed that the injection site was indeed laterally of the PDF-positive somata, (50 ± 20 µm lateral of the somata; Fig. 11a). Importantly, the injection did not hit the PDF-positive somata. We injected three PDF concentrations (0.001 mM, 0.01 mM and 0.01 mM) and repeated each experiment 3 times. Figure 11b shows the pooled data from all three experiments. Control bees that were similarly handled and chilled, but not injected showed a small phase delay of less than an hour on average. The phase delay was longer than 1 hour for the saline and PDF injected bees. Injections of 0.1 mM and 0.001 mM PDF produced a statistically significant longer phase delay relative to un-injected control bees, but only injection of 0.1 mM PDF a larger phase delay compared with saline injection (for statistic results see Figure legend). These results show that both saline and PDF injection at CT8-11 cause a significant phase delay and suggest that the influence of PDF is dose-dependent (this is however not fully clear because the influence of 0.01 mM PDF appeared lower than for 0.001 mM).

The free-running period did not differ between bees injected with PDF, saline, or not-injected in the experiments with the three PDF doses (Two-way ANOVA; experiment with PDF 0.001 mM, $p = 0.514$; PDF 0.01 mM, $p = 0.921$; PDF 0.1 mM $p = 0.094$). The strength of circadian rhythms (power) was lower after injection, even for the control non-injected bees, but this attenuation was not affected by treatment in the experiments with PDF 0.01 mM (ANOVA, $P=0.6$) and 0.1 mM ($p=0.45$). In the experiment with the lowest PDF dose (0.001 mM), the decrease for the control (non-injected) bees was significantly larger (Two-Way ANOVA, LSD post-hoc test, $p<0.05$). Thus, we conclude that the injection of PDF or saline did not affect the strength of circadian rhythms.

2.4.2. Stereotactic injections through the head capsule cuticle without cutting a window

Based on the results of the first set of experiments, we injected a concentration of 0.1 mM PDF in this second set of experiments. In the first trial, the estimated injection sites were mostly located proximally of the PDF somata, only a few may have hit the somata themselves (Fig. 10c), in the second trial they were located closer to the somata (Fig. 10d). There was a strong effect to the estimated injection sites on the measured phase shift. Although in a few bees saline or PDF injection caused a phase advance, in average the injections induced a phase delay which appears larger after injecting PDF compared to saline (but this difference was not significant). In both trials using this protocol, the injection of PDF or saline did not affect the period (FRP) or strength (power) of circadian rhythms in locomotor activity ($P>0.05$, data not shown).

Discussion

The experiments summarised above lend credence to the hypothesis that PDF neurons in the honey bee brain fulfil all required anatomical prerequisites to transfer rhythmic signals to other brain regions, including those involved in time associative memory and time-compensated sun-compass orientation. We show that PDF is most likely rhythmically released into diverse relevant brain regions and that PDF injections can phase shift the circadian rhythm of locomotor activity. Altogether this indicates that PDF plays important roles in the circadian system of the honey bee. These findings with an insect from the

order Hymenoptera are consistent with earlier studies showing that PDF plays a pivotal role in the circadian system of the fruit fly *Drosophila melanogaster (Diptera)*, the cockroach *Rhyparobia maderae* (Blattodea) and the cricket *Gryllus bimaculatus* (Orthoptera).

2.5. PDF fibres pass next to PER expressing neurons and glia cells

The clock protein PER of the honey bee *Apis mellifera* is present in neurons in the lateral and dorsal brain, as well as in numerous glia cells throughout the brain and the optic lobes similar to the distribution in *D. melanogaster* [17,54,55]. Here we show that fibres stemming from the PDF-positive LN_2 come close to most other clusters of PER-expressing neurons, and are accompanied by PER-positive glia cells in most brain regions. This finding is similar to PDF immunostaining in *Drosophila melanogaster*, in which the PDF neurites from the PDF-positive LN_v are accompanied by PER-positive glia cells [16,56] and come close to other PER-positive neurons in the lateral and dorsal brain [57]. In *Drosophila* it was further shown that many of these PER-positive cells also express the PDF receptor [32,58–60] and that PDF signalling strongly affects the oscillations of the other clock neurons and the flies' behavioural rhythmicity [19,29,31,61–70]. Our findings that PDF levels in the honey bee brain cycle over the day, and that injection shifted the locomotion phase, suggest that PDF neurons have similar functions in honey bees and fruit flies. The close proximity of PDF fibres and PER-positive glia in the honey bee is also significant. In *Drosophila* it was shown that electrical manipulation of PER-positive glia cells makes fruit flies arrhythmic, suggesting that glia cells are involved in the clock network and important for rhythmic behaviour [71,72]. The importance of PER-positive glia cells for circadian rhythms was also recently demonstrated in the mammalian circadian clock, the SCN [73].

2.6. Comparison of PDF arborisation pattern in the honey bee brain with that of other insects

PDF immunocytochemistry was performed in the brain of many insects including locusts, cockroaches, crickets, bugs, cicadas flies and bees [8–11,13–15,18,74–76], with the most detailed description available for the cockroach *Rhyparobia maderae* (formerly *Leucophaea maderae*) [18,48,77–80] and the fruit fly *Drosophila melanogaster* [81]. In all these species, with the exception of moths and butterflies [77], PDF-positive somata could be localized to the optic lobes or the lateral protocerebrum [82,83].

2.6.1. Number and size of the PDF neurons

We found on average ~15 PDF-positive somata of different size, which are all located close to the anterior medulla. This number fits to the ~14 PDF somata reported previously for honey bees [13] [14] and the 9-15 PDF somata found in bumble bees [15]. It also roughly fits to the number of ~ 12 PDF-positive somata located anteriorly of the medulla in the cockroach (aPDFMe neurons [77]). Whereas, the cockroach possesses additionally 8 PDF neurons with somata posterior of the medulla and many PDF neurons with small somata close to the dorsal and ventral lamina [48], such PDF-positive somata do not exist in the bee brain. Furthermore, whereas the somata of the cockroach aPDFMe neurons are located at the ventromedial edge of the medulla, the honey bee PDF neurons are located at its dorsomedial edge and have a larger distance to the medulla than the cockroach aPDFMe.

In all species investigated so far the PDF somata vary in size [10,13,14,48,77,84,85]. We observed one particularly large, strongly stained neuron that was localized anteriorly to the other PDF-positive neurons whereas middle-sized and smaller somata were usually located more posterior and closer to the PDF fibre network. In cockroaches and flies, the PDF neurons with large somata appear to show wide field arborisations that span the entire brain and connect both brain hemispheres [57,79,84]. In honey bees we could not assign fibres to individual neurons, but similar to cockroaches and flies the large and middle-sized honey bee PDF neuron may arborize throughout the entire brain, whereas the ones with small soma may remain more local within the ipsilateral hemisphere.

2.6.2. Is there an accessory medulla (AME) in the honey bee?

The AME can be regarded as the most important communication centre in the circadian network of insects, which additionally gets input from external Zeitgebers (environmental time cues). This small neuropil at the base of the medulla is typically characterized by a dense network of PDF-positive fibres. It is best characterized in the cockroach *R. maderae*, where it is densely innervated by PDF-positive and other peptidergic neurons that may be similarly engaged in the circadian clock [77,79,80]. It is organized into a nodular core receiving photic input from the eye and into an internodular and peripheral neuropil involved in efferent output and coupling input from other clock neurons [77,80]. In *D. melanogaster*, the AME is less conspicuous, but as in the cockroach it receives dense input from the PDF-positive neurons as well as from the majority of other clock neurons and it is innervated by an extra retinal eye, the Hofbauer-Buchner eyelet [85–87].

We identified a small neuropil at the base of the honey bee medulla which is even less noticeable than the AME of flies and is not densely innervated by PDF-positive fibres. Interestingly, an area with much higher density of PDF fibres was found proximally of the PDF neurons in front of the lobula that

we call here ALO. Thus, we suggest that the ALO is a more important communication centre of the honey bee circadian clock neurons than the AME. A similar located PDF-rich network was also described for the bumble bee, *Bombus terrestris* [15]. We suggest that the bee ALO is not anatomically homologous to the AME of the cockroach or the fly. Nevertheless, the ALO of bees is located very close to the somata of the PER-positive LN_1. In *Drosophila* several lateral neurons are in close vicinity of the AME and the majority of the other PER-positive clock neurons project into the AME. Additional studies are needed in order to determine whether the bee LN_1 and the other PER-positive clock neurons do also project into the ALO.

The apparent neuroanatomical differences in the circadian clock centre of bees and other insects may be explained by developmental processes that we discuss in the supplementary material. We assume that honey bees possess an AME, but that, during development, a close association with photoreceptors is lacking and consequently a dense innervation by PDF fibres is missing. It will be interesting in the future to perform developmental studies in order to see how the PDF neurons and their arborisations look during larval and pupal stages.

2.7. Connections of the PDF neurons with higher integration centres in the brain

Both time memory and sun-compass navigation require that information about time of day is conveyed to brain structures responsible for memory and sun-compass orientation. Below we discuss whether PDF neurons fulfil the anatomical criteria to provide such an input.

2.7.1. Connections between PDF neurons and memory centres in the mushroom bodies

Honey bees are excellent learners, quickly forming associations between stimuli of different sensory modalities [88] and showing various forms of conceptual learning [89] that are largely mediated by their highly developed mushroom bodies. The calyces of the mushroom bodies (MCA and LCA) are large and receive input from olfactory and visual cues [90], whereas the vertical lobes are thought to be the main output regions of the mushroom bodies [91]. We found that PDF neurons do not invade the mushroom body neuropils, but rather wrap their various parts. The densest network of PDF fibres is found ventral to the lateral and median calyces of the mushroom bodies, but also the vertical, median lobes and the peduncle are surrounded by varicose neurites of PDF neurons. For example, the PDF fibres form rings around the vertical lobe and the peduncle (see Fig. S4). Since PDF is most likely stored in the varicosities and released from there (and other parts of the neurite) in a paracrine manner, it likely can reach into the mushroom bodies and convey time-of-day information, and this appears possible in many places.

2.7.2. Connections between PDF neurons and the sun-compass pathway

The neuronal basis and mechanisms underlying sun-compass orientation have been investigated in detail in locusts (reviewed by [92,93]). Two major pathways that transfer sun compass signals to the central complex - a prominent anterior one and a less striking posterior one – have been described [94]. Recently, three papers have been published indicating that at least the anterior polarization vision pathway is conserved between honey bees, locusts, bumble bees and ants [50,95,96]. The sun-compass pathway receives skylight polarization input from a specialized area of the compound eye, the dorsal rim area. Photoreceptors in this area project through the dorsal lamina and terminate in the dorsal rim area of the medulla, which is called "MEDRA". The MEDRA is innervated by transmedulla neurons that carry the polarized light information through the serpentine layer of the medulla and then via the anterior optic tract to the anterior optic tubercle [95]. From there, interneurons make a connection to the lateral and medial bulbs (MBU and LBU) [95,97]. In the bulbs, they form conspicuous large synapses with GABA-ergic tangential neurons of the central body's lower division (CBL) [50]. These neurons are then connected to the protocerebral bridge of the central complex which holds a topographic representation of zenithal polarization angles [98–100]. The possible posterior polarized-light input pathway start also from the serpentine layer of the medulla and ends in the protocerebral bridge; but it passes via the AME and the POC to the POTU that locate adjacent of the most lateral endings of the protocerebral bridge. Output neurons from the protocerebral bridge project to the lateral accessory lobe (LAL [94,100,101]) and finally, polarization information is send via descending neurons to thoracic motor control centres [102]. In honey bees, the pathways within the central complex as well as the output pathway to the lateral accessory lobe are not yet clarified, but are likely to be similar.

We find PDF-positive fibres (1) in the dorsal rim area of the lamina, (2) between the dorsal rim area of the lamina and medulla and (3) in the dorsal rim area of the medulla. These fibres extend along the projections of the dorsal rim photoreceptors that run into the MEDRA. Our results are in line with the data of Zeller et al. [95], who combined PDF immunocytochemistry with tracing of sky compass pathways to the MEDRA that is surrounded by PDF fibres. They further show that the PDF fibres in the serpentine layer of the medulla overlap with the pathway of the transmedulla neurons that run via the anterior optic tract into the central brain. Consistent with their observations, we do not see any PDF-

positive fibres in the anterior optic tract and the anterior optic tubercle, but we see a dense PDF fibre network directly behind the anterior optic tubercle. In addition, we see a ring of PDF fibres around the vertical lobes, exactly at the location where the interneurons from the anterior optic tubercles take their path toward the bulbs (MBU and LBU). The bulbs themselves are not innervated by PDF fibres, but again, PDF varicosities are very close to them. We also see a dense PDF network in the median accessory lobe (MAL), a brain area that seems to be associated with the central complex [103] and PDF fibres in an area that resembles the putative lateral accessory lobe (LAL). The boundaries of the honey bee LAL, are less well defined and therefore we cannot be sure, whether the PDF fibres also enter the LAL, or just pass close to it. Concerning the possible posterior polarized-light input pathway, we found PDF fibres that leave the POC and may innervate the POTU. Thus, here might be a direct input from the PDF neurons.

In summary, we see a close vicinity of PDF fibres to the sky-compass pathway, not only on its input side to the central complex, but also on its putative output pathway. Thus, a rhythmic paracrine release of PDF in several places can potentially convey time-of-day information to the sun-compass network.

2.8. PDF oscillations

The transfer of time-of-day information from the LN_2 neurons to PDF-responsive brain structures requires a time-dependent release of PDF. Indeed, we detected oscillations in PDF staining intensity in all analysed brain areas. These oscillations are consistent with the hypothesis that PDF is rhythmically released into the protocerebrum and the medulla of the bee. In all the experiments, PDF-staining intensity showed a clear trough during the early day, and highest intensity during the night. The timing of PDF oscillation in the honey bee differs from that reported for *D. melanogaster*, in which PDF staining intensity in fibres terminating in the dorsal protocerebrum was maximal in the morning and lowest during the night [26,28]. In addition the *Drosophila* PDF terminals showed more branching in the morning, when PDF is transported to the terminals and released into the protocerebrum, than in the evening [27]. Our analyses for the honey bee are not consistent with this mechanism. PDF was always present and we did not notice significant daily changes in the varicosities and the branching, although we may have overlooked minor changes. We thus suggest that in the honeybee PDF release does not depend as much on a rhythmic transport into the terminals as it does in *Drosophila*. A more likely explanation is that PDF is stored in the varicosities, and when it is released, PDF staining intensity drops temporarily. This kind of rhythm would also fit to the rhythm in honey bee *Pdf* mRNA, which shows a maximum at the end of the day, after PDF has been released from the terminals and starts to increase again and a minimum during the beginning of the night, when PDF levels have reached their maximum in the terminals [14]. In *Drosophila*, no cycling in *Pdf* mRNA levels has been observed [26], consistent with the premise that the PDF rhythm relies mainly on rhythmic transport of PDF into the terminals and not on rhythmic synthesis. The fact that PDF levels in *Drosophila* cycle in opposite phase in the somata and the terminals of the neurons support this idea. In the honey bee, on the other hand, PDF cycling in the somata and in the fibres are in phase with each other and the observed cycle seems to depend on rhythmic synthesis and release. If our hypothesis is true, PDF would be released in the morning from the terminals of both diurnal insects.

2.8.1. PDF levels oscillate in both foragers and nurses

Nurses are active around-the-clock with attenuated circadian rhythms in locomotor activity and in whole-brain *per* mRNA abundance [13]. However, some of their pacemakers generate endogenous rhythms and can be entrained by social Zeitgebers [17,104–106]. Consistent with this evidence for functional clocks, we found here that also the PDF staining intensity oscillates in the nurse brain. Nevertheless, our analyses suggest that there are some differences between nurses and foragers in the degree of synchronisation among the brain sites. In nurses, PDF oscillations in the different brain areas that we measured appear to be better synchronized, than in foragers. Although our sample size is quite limited at this stage (three colonies for foragers, and two for nurses) this finding deserves attention.

The lower synchrony in foragers may stem from their exposure to a more diverse set of external Zeitgebers (time-givers) than nurses. The nurses were directly collected from the hive and did not experience any light-dark or significant temperature cycles. Their circadian clock was probably entrained mostly by social cues (coming from the strongly rhythmic foragers) [53,105]. Another, not mutually exclusive explanation for the differences in synchrony is that the circadian system of nurses is not yet mature, and that in the mature system of foragers, PDF is released with different kinetics and at slightly different times into the diverse brain areas.

It is too early to speculate about the biological function of this apparent task-related variability in synchrony in PDF rhythms across the brain, but rhythms with different phases are common in the brain of mammals [107]. clock neurons in different parts of the brain show differently phased Ca^{2+} rhythms [30,108]. Likewise, such differently phased rhythms may occur in the brain of honey bees.

2.8.2. PDF cycling continues under constant darkness

PDF cycling in the honey bee seems to continue under constant darkness, at least in the serpentine layer of the medulla. Although our statistical analysis did not reveal significant time-dependent differences in PDF staining intensity in the LVT and ventrally of the calyces, there was a clear trend of cycling with an amplitude of two to almost four-fold change also in these brain areas. We suspect that because these bees were sampled on the fourth day of DD, the oscillations in individual bees differing in their free-running period were too much out of phase, and the variation in each time point was very high. However, it is notable that even after three days of free-run the trough still occurred on the early subjective morning, which is consistent with the pattern that we found in the other experiments.

2.9. PDF injections phase shifted circadian rhythms in locomotor activity

We used two different protocols to test the influence of PDF injection on circadian rhythms. With both protocols saline injection into the area between the optic lobes and the central brain caused a strong phase delay. This strong effect is consistent with the neuroanatomical studies that show that this brain area is adjacent to the LN_1 and LN_2 PER-positive clusters and is rich in PDF expressing fibres that originate in the LN_2 neurons. PDF injection caused a dose-dependent phase-delay, when injected in 20-50 µm distance of the PDF neurons during the early night (using the protocol in which we opened the head capsule). We received an overall similar effect with the second protocol, in which we did not open the head capsule, although the difference between PDF and saline injection was not statistically significant. We assume that this is because of the higher spatial variability of this protocol, and the fact that the PDF effect differs between sites of injection. The finding that PDF injection phase shifted circadian rhythms fits with similar findings for cockroaches and crickets lend credence to the hypothesis that PDF is an integral part of the circadian neuronal network [33,35]. It would be interesting to know whether the PDF effect is time-dependent with a phase advance at some parts of the circadian cycle, as it has been demonstrated for cockroaches and crickets. With both protocols PDF injection into one side of the brain did not affect the strength (power) or period of circadian rhythms in locomotor activity. These findings suggest that PDF injection did not modify the pace of oscillation in key pacemakers that control circadian rhythms in locomotor activity. The lack of effect on the strength of circadian rhythms suggest that the injection did not disturb the phase relationship between the various PDF responsive neurons that are involved in the regulation of circadian rhythms in locomotor activity.

In sum, our detailed neuroanatomical descriptions reveal that PDF fibres stemming from the LN_2 clock neurons arborize extensively in the optic lobes and central brain of the honey bee. PDF fibres reach many PER positive neurons and glia cells and cross to ipsilateral parts of the brain, which is consistent with a role in coupling the different components of the brain circadian system. PDF fibres are also excellently positioned to function in both input and output pathways of the circadian clock. These include extensive arborisation in the optic lobes that integrate visual information and the dorsal rim area that is sensitive to light polarization and important for sun-compass orientation. The extensive arborisations in the central brain are excellently positioned for conveying time-of-day information from the LN_2 to brain centres involved in various clock output functions. These include neuropils involved in locomotor activity, sun-compass orientation and time-associative learning. The evidence that PDF levels show a clear trough during the early day (or subjective day in constant darkness) suggests that the timely release of PDF is a mechanism to convey time-of-day information to target neurons expressing the PDF receptor. This premise is further supported by our injection studies that show that an artificial elevation of PDF levels phase shifts circadian rhythms in locomotor activity. This study together with our earlier characterization of PER immunostaining [17] provides the best description of the bee circadian network available so far and sets the stage for studies on the interplay between the circadian clock and complex behaviours such as division of labour, dance communication, sun-compass orientation, and time memory.

Ethics statement. Not applicable.
Data accessibility. A movie showing a 360° turn of the 3-D PDF network reconstruction in the honey bee brain is available as supplementary material (AVI-file plus Tiff-file) together with several supplementary figures and an extended discussion (Word-file).
Competing interests. We declare no competing interests.
Authors' contributions. K.B. performed the double-labelling with anti-PDH and anti-PER as well as the triple- or quadruple-labelling experiments with anti-PDH, anti-PER, anti-HRP and DAPI on whole-mount brains and sections, analysed staining patterns, made reconstructions of PER and PDF double stained brains with AMIRA, finalised the PDF reconstructions in AMIRA, produced the movie and wrote parts of the paper. E.K. performed anti-PDH and Lucifer yellow staining, performed the PDF reconstructions in AMIRA, and performed and analysed the PDF cycling experiments in Germany. N. K.

and N. Y. performed and analysed the PDF-injection experiments. R. W. performed the PDF cycling experiments in Israel and helped with immunostaining and analysis. P.M. established and supervised several staining protocols and performed preliminary PER and PDF double-labelling experiments. G.B. conceived the project, contributed funding, guided research and contributed to the writing of the paper. C.H.-F. conceived the project, contributed funding, guided research, composed the pictures and wrote the paper.

Acknowledgments. We thank Eva Winnebeck for kindly sharing the antibody against the PER protein and Heiner Dircksen for the anti-PDH antibody. We would like to thank Angelika Kühn for technical assistance with the bee experiments at the University of Regensburg and the Department of Animal Ecology and Tropical Biology, University of Würzburg, for providing honey bees in Würzburg. We are furthermore thankful to Keram Pfeiffer and Basil el Jundi for help with the neuroanatomy of the honey bee brain, Jürgen Rybak and Annekathrin Lindenberg for help with AMIRA, Frank Schubert for advice on reconstruction methods, Monika Stengl for teaching us her injection protocol and Binyamin Hochners for sharing the Diel fluorescent dye. We also thank Hadas Lerner, Ravid Shahar, Talya Kerem and Jeffrey Jackson for assistant with the injection and immunocytochemichal studies in Israel, to Rafi Nir for beekeeping the honey bee colonies in Israel, and for Mira Cohen for technical assistant in Israel. Finally, we thank Basil el Jundi and Wolfgang Rössler for critical comments on the manuscript.

Funding Statement. This research was supported by funds from the German Israeli Foundation for Scientific Research and Development (G.I.F. project number 1-822-73.1/2004) to G.B and C.H.-F., the German Research Foundation (CRC 1047 "Insect Timing", project A1) and Israel Science Foundation (ISF; project number 15/1274) to G.B, and two short-term travel grants from the Minerva foundation (to R.W. and N. K.).

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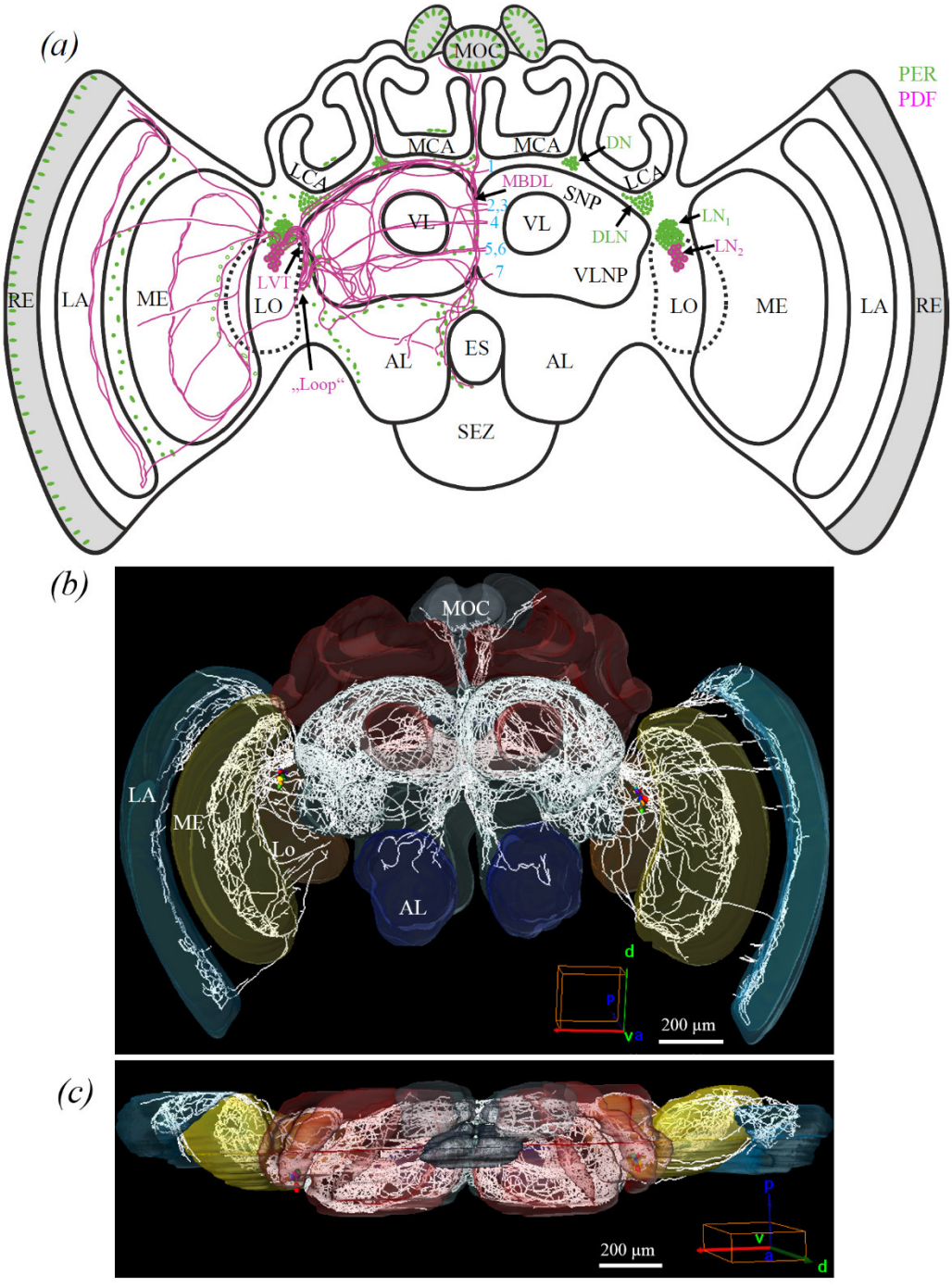

Figure 1

Figure 1. PER- and PDF-positive cells in the brain of the honey bee *Apis mellifera*. *(a)* Schematic presentation of the PER (green) and PER/PDF (magenta) -positive cells in the brain. The main fibre tracts of the PER/PDF-positive neurons (magenta) are additionally depicted in the left brain hemisphere together with the nuclei of PER-positive glia cells and photoreceptor cells (green). LN_1 lateral neurons 1, LN_2 lateral neurons 2, DLN dorsolateral neurons, DN dorsal neurons. ***(b,c)*** Reconstruction of all PDF fibres which emanate from the PER-/PDF-positive LN_2 in a frontal (anterior) view ***(b)*** and from a dorsal view ***(c)***. Cell bodies are coloured, fibres are depicted in white. The PDF fibres invade all optic ganglia (LA lamina, ME medulla, LO lobula), the ocelli (MOC medial ocellus) and most parts of the bee's protocerebrum, as well as sparsely the antennal lobes (AL) of the deutocerebrum. In addition, the tritocerebrum shows PDF-positive fibres around the oesophageal foramen that are hard to see in these reconstructions. Mushroom bodies and the central complex are free of PDF fibres. AL antennal lobe, LA lamina, LO lobula, ME medulla, RE retina, SEZ

subesophageal zone, SNP superior neuropils, VLNP ventrolateral neuropils, LCA lateral calyx, MCA medial calyx, VL vertical lobe.

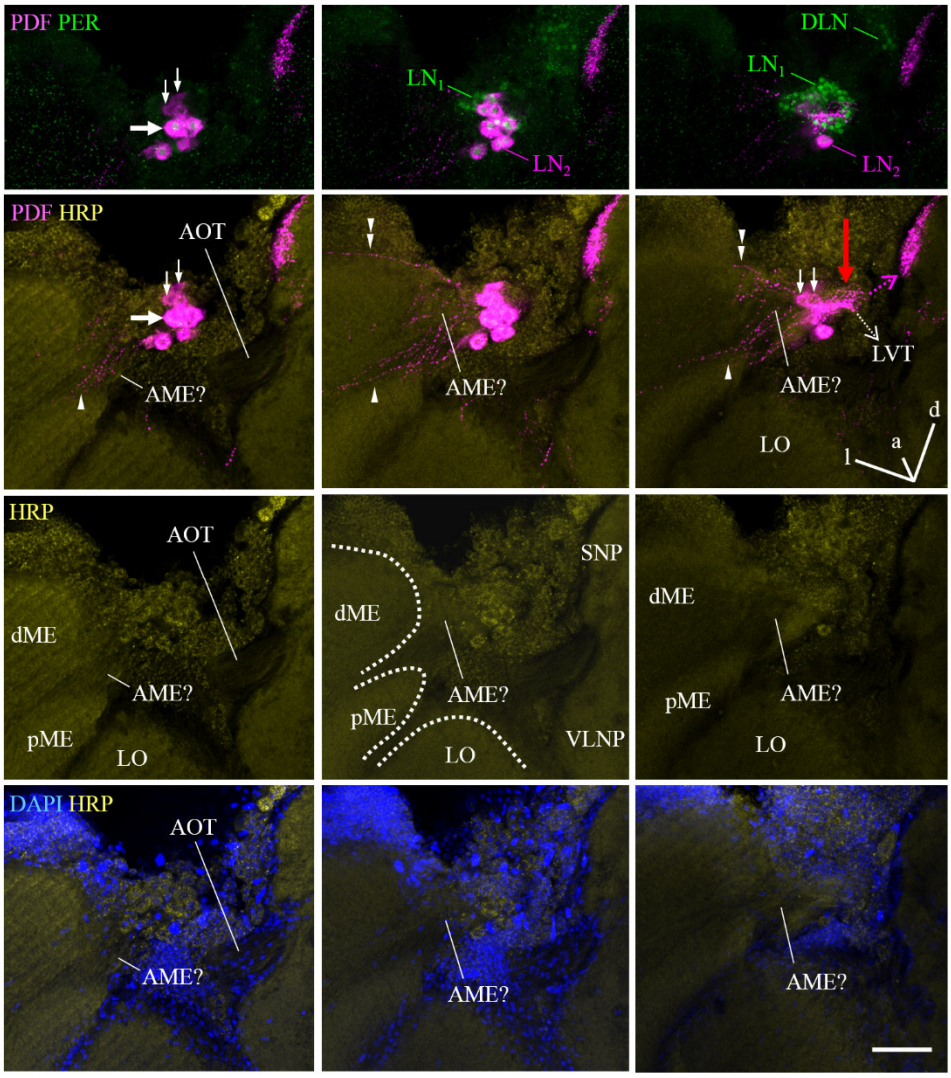

Figure 2

Figure 2. Fibers arising from the PDF-positive somata. Overlays of 6 confocal stacks, each, in the anterior (left), medial (middle) and posterior (right) area around PDF-positive somata of a 60 µm thick semi-frontal vibratome section (left hemisphere). Note that this section is tilted in the anterior-posterior as well as in the left-right plane to visualize the serpentine layer of the medulla. The section is labelled with anti-PER, anti-PDF, anti-HRP and DAPI. The upper row shows PER and PDF labelling in the dorsal region of the pictures to demonstrate the vicinity of the LN_1 and LN_2 (compare Fig. 1*a* and 3). In this row the PDF-positive neurites arising from the LN_2 are hard to see, because we reduced PDF labelling intensity. The large arrow points to the largest PDF neuron, the small arrows to the smallest PDF neurons. The second row shows PDF labelling at higher intensity in order to reveal the arborisations of the LN_2 on top of HRP labelling. HRP labelling alone is shown in the third row. HRP visualizes the major neuropil regions (plus the somata of neurons): dME distal medulla, pME proximal medulla, LO lobula, small neuropil reminiscent of the accessory medulla (AME?), SNP superior neuropils, VLNP ventrolateral neuropils). The neuropils are free of somata and nuclei as can be seen by the combination of DAPI and HRP labelling in the lowest row. Large tracts, as the anterior optic tract (AOT) usually contain the nuclei of glia cells, whereas the putative AME is basically free of nuclei. The PDF neurons send sparse neurites into the putative AME and from there into the serpentine layer of the medulla (arrowhead) and to a minor degree into the most distal layer of the medulla (double arrowhead). Proximally they send many fibres with dense varicosities onto the surface of the lobula (red arrow) and from there to the superior neuropils (SNP) (magenta broken arrow). From the densely labelled fibres anterior of the lobula (ALO: anteriolobular PDF hub) the lobula valley tract (LVT)

originates and projects posteriorly and ventrally along the inner surface of the lobula (broken white arrow; for details on the LVT see Fig.4). Please note that the AOT is free of PDF fibres. Scale bar: 50 µm; orientation arrows: l: lateral, a: anterior, d: dorsal

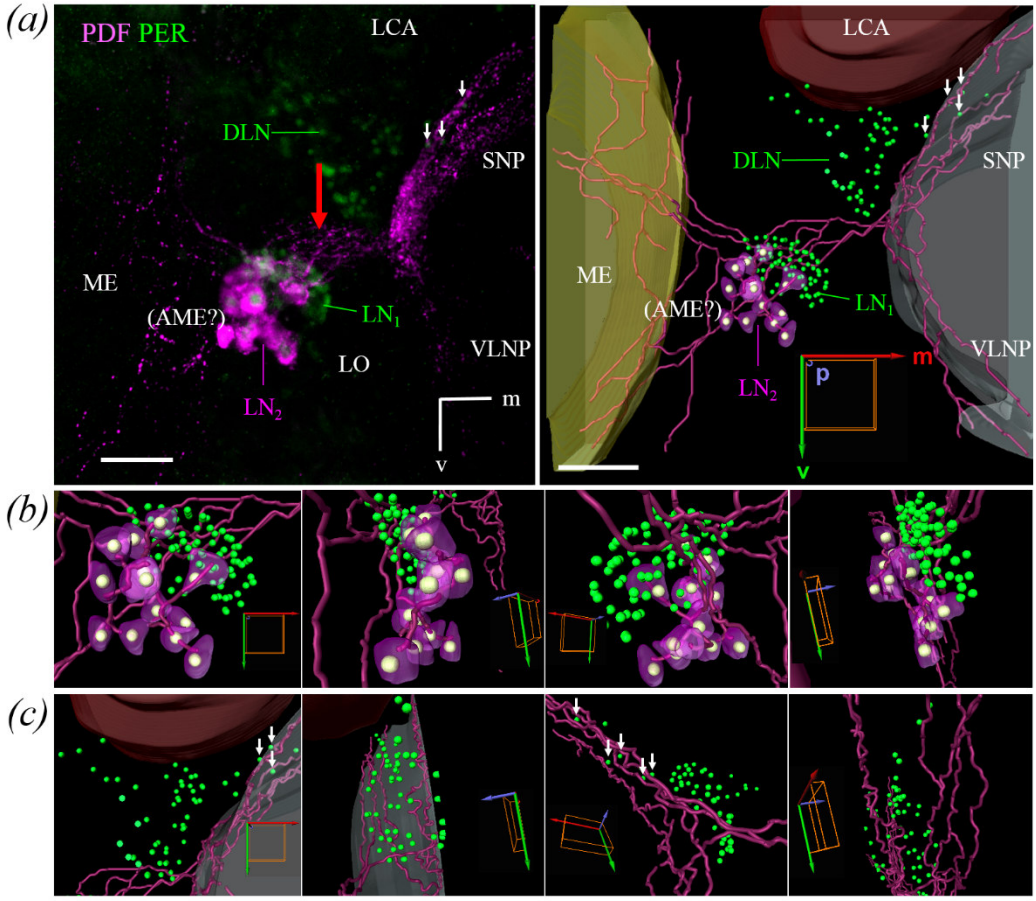

Figure 3

Figure 3. Vicinity of the PER-positive LN_1 and DLN clusters to PDF-positive fibres originating from the LN_2 cluster. (***a***) Projection picture of several layers scanned though the left lateral part of a whole-mount honey bee brain (left) and relevant reconstruction of this part in Amira (right). PDF (magenta) is present in the cytoplasm of the LN_2 cluster and labels additionally its neurites, whereas PER (green) labels the nuclei of all three cell clusters (DLN, LN_1 and LN_2). The nuclei of the LN_2 cluster are shown in white in the reconstruction. Note that the PDF neurites running to the putative accessory medulla (AME?) and medulla (ME) are less dense than the PDF neurites running towards the superior (SNP) and ventrolateral neuropils (VLNP). The LVT is not yet visible at this anterior level. The PDF-positive neurites from the LN_2 at the surface of the lobula (red arrow, described as ALO (anteriolobular PDF hub) in the text) pass the LN_1 in close proximity. Furthermore, the ones running into the SNP are close to a few DLN neurons (small white arrows). (***b***) Larger magnification of the reconstructed LN and (***c***) DLN clusters viewed from anterior, lateral, posterior and medial, respectively. Scale bars: 50 µm. LCA: lateral calyx; orientation arrows: m: medial, p: posterior, v: ventral

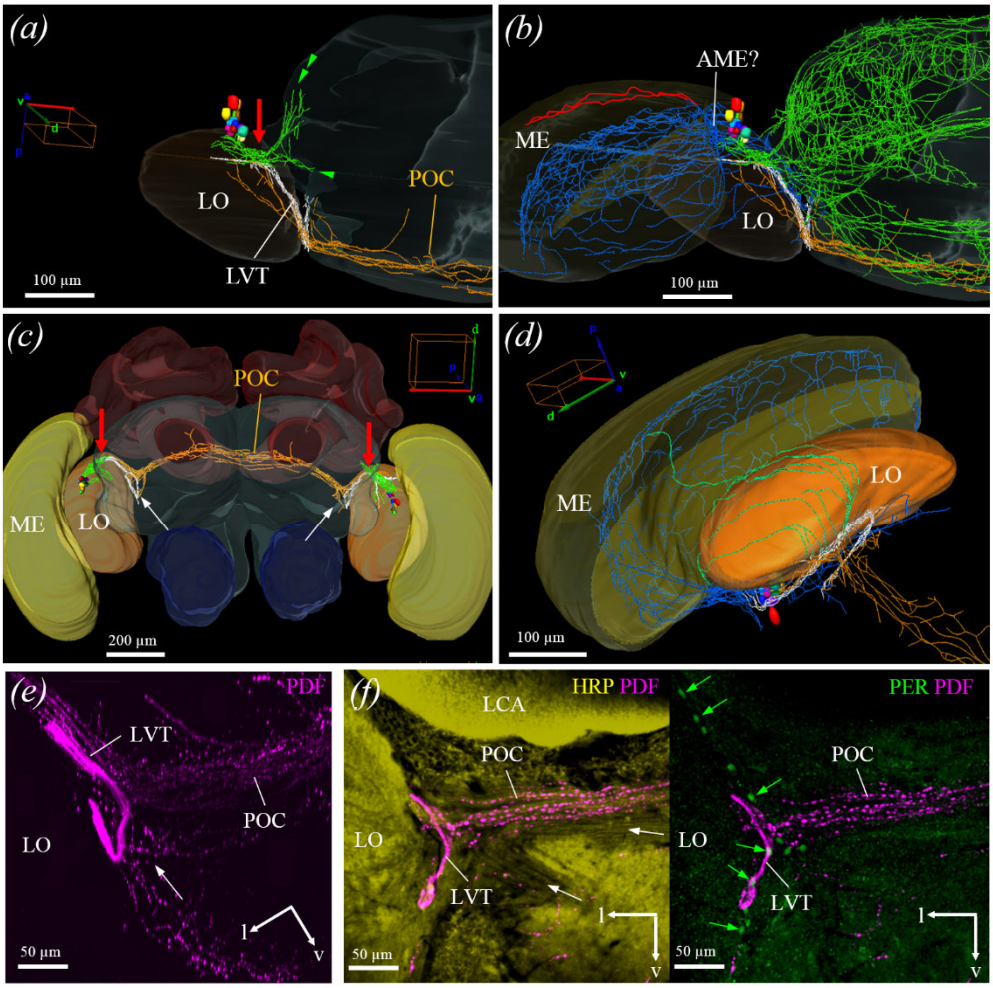

Figure 4

Figure 4. The PDF-positive lobula valley tract (LVT) and the main fibre tracts arising from it. (*a*) Reconstruction of the PDF somata and the fibres arising from them from a dorsal view of the left brain hemisphere, medulla (ME) and lobula (LO). The LVT (white fibres) originates from the dense PDF fibres on the anterior surface of the LO, called ALO (anteriolobular PDF hub, red arrow) and runs posteriorly and ventrally always remaining on the surface of the LO. On its way, many fibres leave (or enter) the LVT: fibres toward the anterior surface of the protocerebrum (green double arrowhead; e.g. the SNP and VLNP as shown in Fig. 3), fibres toward the median protocerebrum (green single arrowhead), fibres toward the LO (orange) and fibres to the posterior optic commissure (POC; orange). (*a*) shows only the main origin of the fibres running toward the protocerebrum, whereas the (*b*) shows all fibres in the protocerebrum (green). In addition it shows the fibres in the serpentine layer of the medulla (blue) and those on its distal surface that innervate the lamina (red). (*c*) gives an overview on the course of the POC from an anterior view. In addition the characteristic loop of the LVT at the posterior rim of the LO can be seen (white arrows). The PDF fibres in the ALO, from which the LVT starts, are again marked by red arrows. (*d*) is a magnification of the LVT course showing its characteristic loop and additional fibres (green) that originate from it and run distally along the posterior surface of the LO. (*e, f*) overlay of several confocal pictures depicting the LVT at its characteristic loop plus PDF-positive fibres in the POC and in a more ventral tract that seems to start close to the loop (white arrow). Please note the many varicosities in the POC and the other tract, whereas the LVT contains virtually no varicosities in this place. (*e*) shows only PDF labelling (overlay of 6 confocal stacks of a whole-mount brain). (*f*) vibratome section labelled with PDF, HRP and PER (overlay of 5 confocal stacks). The overlay of HRP and PDF staining shows other fibre tracts in addition to the POC (white arrows). The right picture shows PDF and PER overlaid. PER is present in several glia cells (no HRP present in their cytoplasm) that are aligned at the border of the LO (green arrows) and at the origin of the POT.

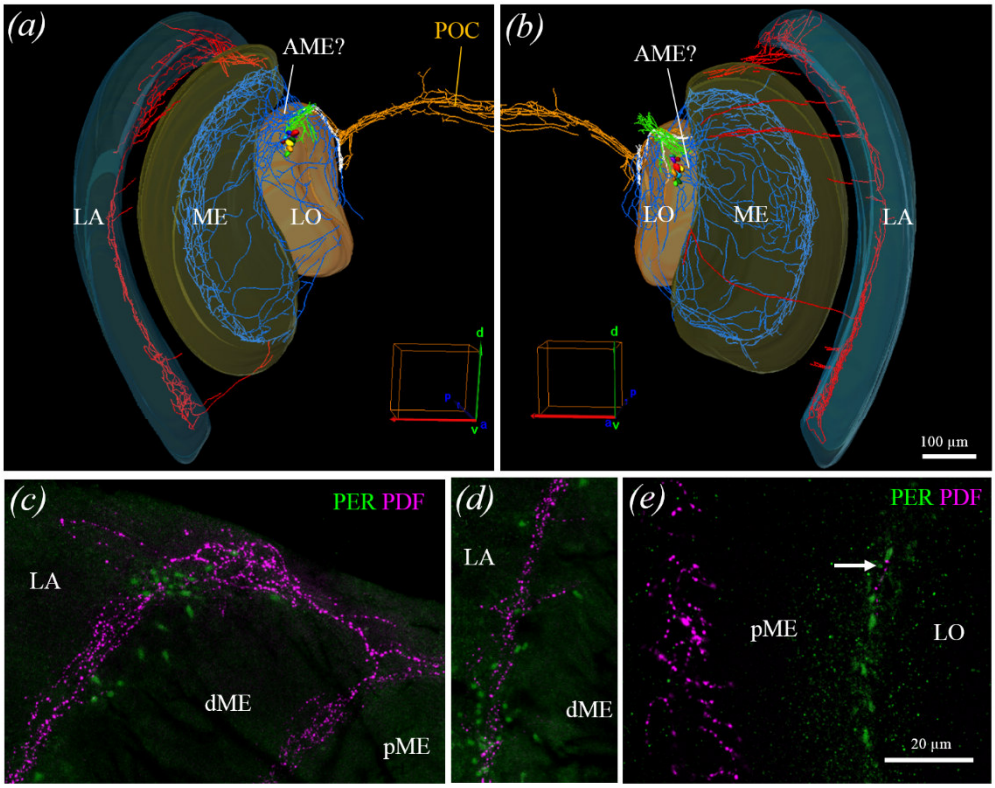

Figure 5

Figure 5. PDF fibres in the medulla and lamina. The upper row shows reconstructions of both optic lobes. (*a*) PDF fibres running to the lamina (LA) are mainly present in the dorsal medulla (ME) and to a lesser extent in the ventral ME. (*b*) the fibres running to the LA are also most prominent in the dorsal optic lobe, but in addition, fibres projection in an anterior fan over the distal surface of the ME to the LA are more frequent. (*c-e*) magnifications of PER/PDF double labelled vibratome sections in the optic lobes. (*c*) dorsal part of the LA and ME (overlay of 6 confocal stacks). Note that the fibres entering the proximal LA are in close vicinity to PER-positive glia cells. The ME is divided into proximal (pME) and distal (dME) part, which are separated by PDF-positive fibres in the serpentine layer. In the serpentine layer only few PER-positive glia cells are located. (*d*) The PDF fibres in the more ventral part of the LA are also accompanied by PER-positive glia cells (overlay of 6 confocal stacks). (*e*) PER-positive glia cells between ME and LO (overlay of 3 confocal stacks). In this region only sparse PDF innervations are found (white arrow).

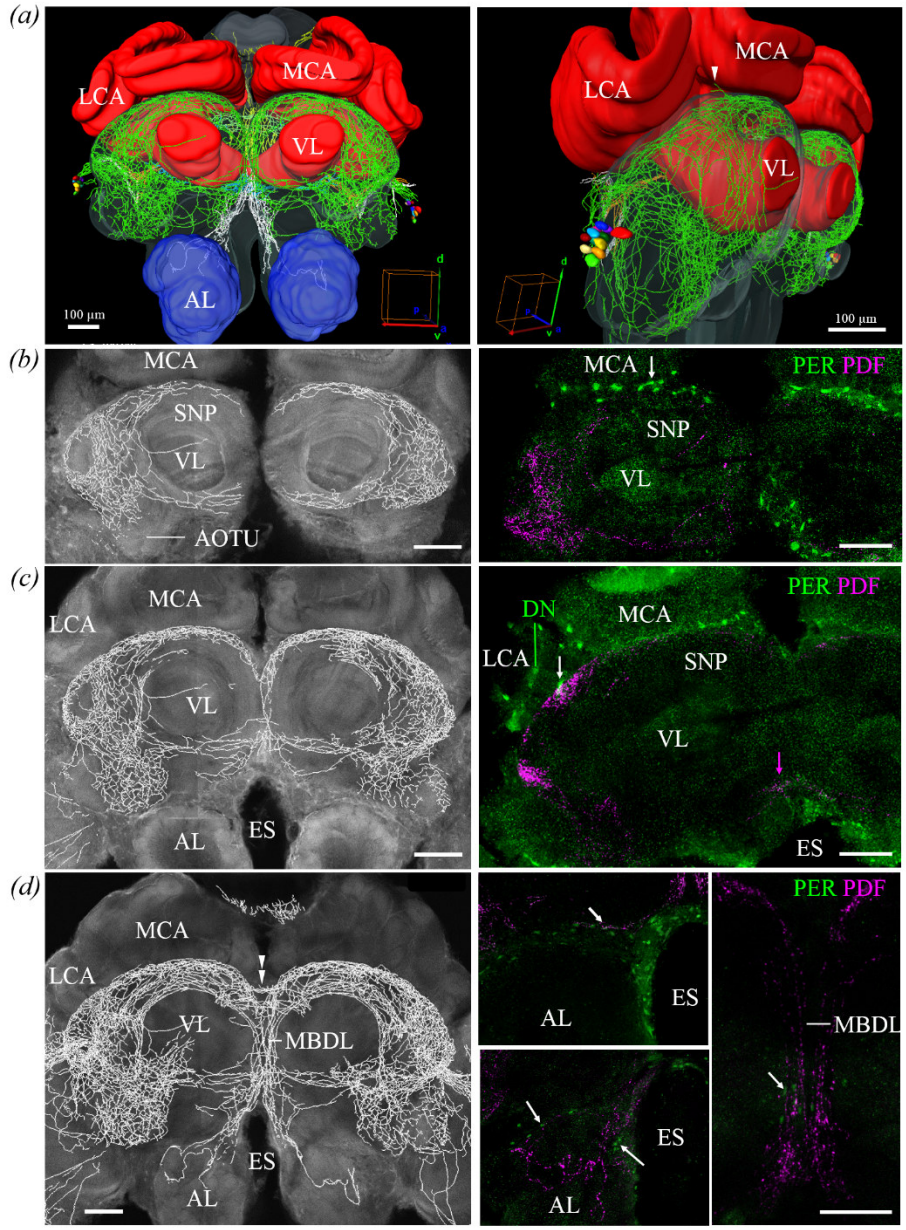

Figure 6

Figure 6. PDF fibres and PER-positive cells in the proto- and deutocerebrum.
(a) The PDF fibres form a network on the entire surface of the protocerebrum (green fibres) that reaches via the median bundle (MBDL in *d*) also the antennal lobes (AL) of the deutocerebrum. The median (MCA) and lateral calyces (LCA) as well as the vertical lobes (VL) of the mushroom bodies are embraced but not entered by PDF fibres. The same applies for the anterior optic tubercles (AOTU) *(b)*. *(b)-(d)* frontal optical sections of the brain from anterior to posterior. The pictures on the left side represent reconstructions of the PDF fibres that are overlaid on the neuropil staining, the pictures on the right are confocal pictures of vibratome sections labelled for PER and PDF at a corresponding depth. Most anteriorly *(b),* PER-positive glia cells (white arrow) are aligned in a row between the MCA and the superior neuropils (SNP). A bit deeper in the brain (*c*), PDF fibres running underneath the LCA and MCA touch PER-positive glia cells (white arrow). Other PDF fibres (not seen here) come close to the PER-positive dorsal neurons (DN). In the ventral protocerebrum just ventrally of the VL, PDF fibres cross the midline of the brain in an anterior ventral commissure (magenta arrow). ES oesophageal foramen. Still deeper in the brain *(d)*, PDF fibres cross the midline in an anterior dorsal commissure (double arrowhead), others run ventrally in the median bundle (MBDL). PDF fibres in the MDBL that are accompanied by PER-positive glia cells (white arrow in the picture to the most right) extend ventrally into the dorsal AL (middle lower picture). Again they are close to PER-positive glia cells (white arrows). At a slightly more anterior level (upper middle picture), PDF-positive fibres run together

with PER-positive glia cells along the ventral border of the protocerebrum (white arrow). Scale bars: 100 µm.

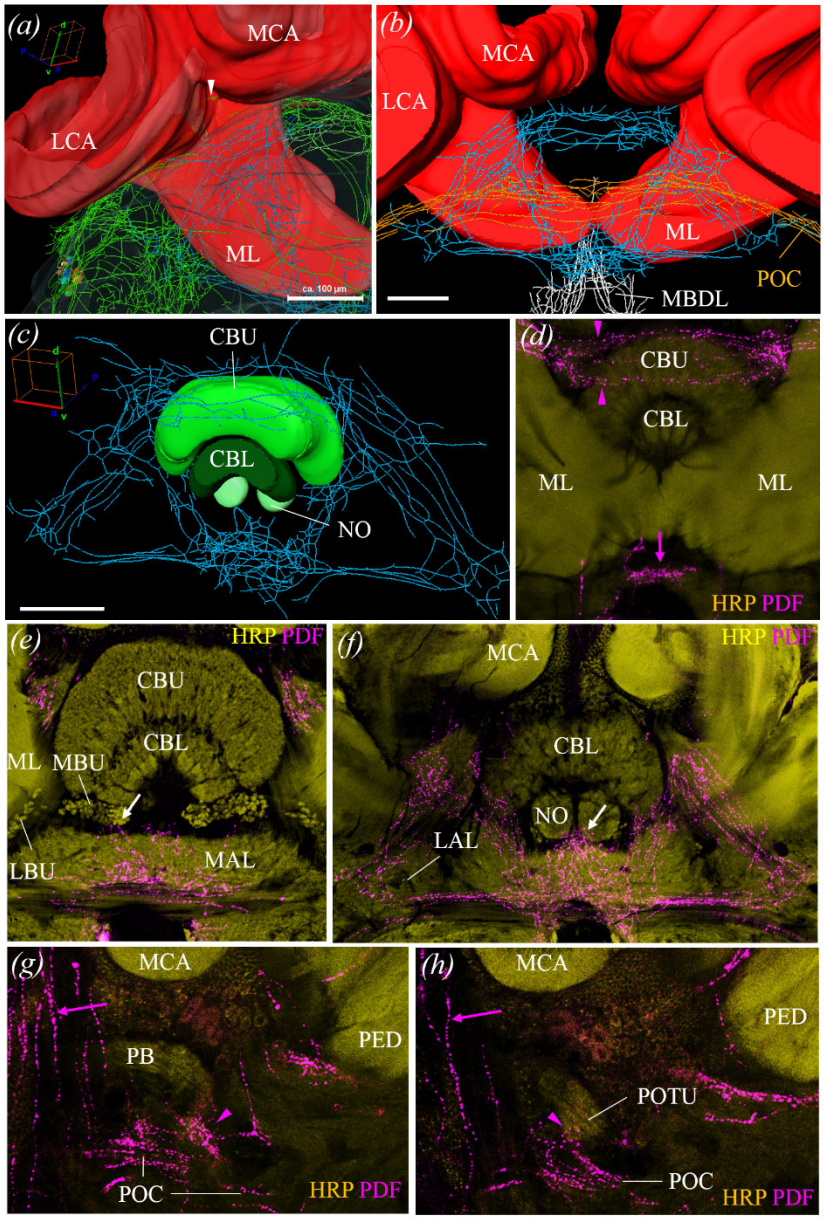

Figure 7

Figure 7. PDF fibres and PER-positive cells posterior of the mushroom bodies, around the central complex and in the posterior optic tubercles. (*a-b*) PDF fibres posterior of the mushroom bodies. The white arrowhead marks the two PDF fibres that pass between the lateral and medial calyx (LCA and MCA) from the anterior to the posterior protocerebrum. The fibres that compose the conspicuous three-triangle network just posterior of the medial lobes (ML) are shown in blue, the ones of the posterior optic commissure in orange (POC); fibres in the anteriorly located median bundle (MBDL) are depicted in white. (*c*) Central complex with the upper and lower division of the central body (CBU and CBL) and the noduli (NO) from anterior. The protocerebral bridge is omitted. The CBU is loosely wrapped by PDF fibres crossing the midline in two commissures. (*d*) PDF fibres in the dorsal (magenta arrow heads) and ventral (magenta arrow) double commissures at the most anterior level of the mushroom bodies at which the two MLs touch each other in the midline of the protocerebrum. (*e-f*) PDF fibres at the levels of the central and lateral complex, respectively. (*e*) PDF fibres arborize in the median accessory lobe (MAL) and extend to the medial bulbs (MBU) of the lateral complex that are characterized by a glomerular structure (white arrow). The lateral bulbs (LBU) of the lateral complex are free of PDF fibres. (*f*) The triangles formed by PDF fibres and described in the text. The white arrow point to PDF fibres that touch the noduli. (*g-h*) PDF fibres running behind the triangle network. The magenta arrows point to PDF fibres leaving the POC and projecting to the ocelli (see also Fig. S6). (*g*) PDF fibres leaving the POC terminate close to the posterior part of the protocerebral bridge

(PB) (magenta arrow head). (*h*) The right posterior optic tubercle (POTU) is innervated by PDF fibres (magenta arrow head) stemming from the POC. All 5 confocal pictures are overlays of 3 confocal stacks. PED: peduncle, Scale bars: 100 µm

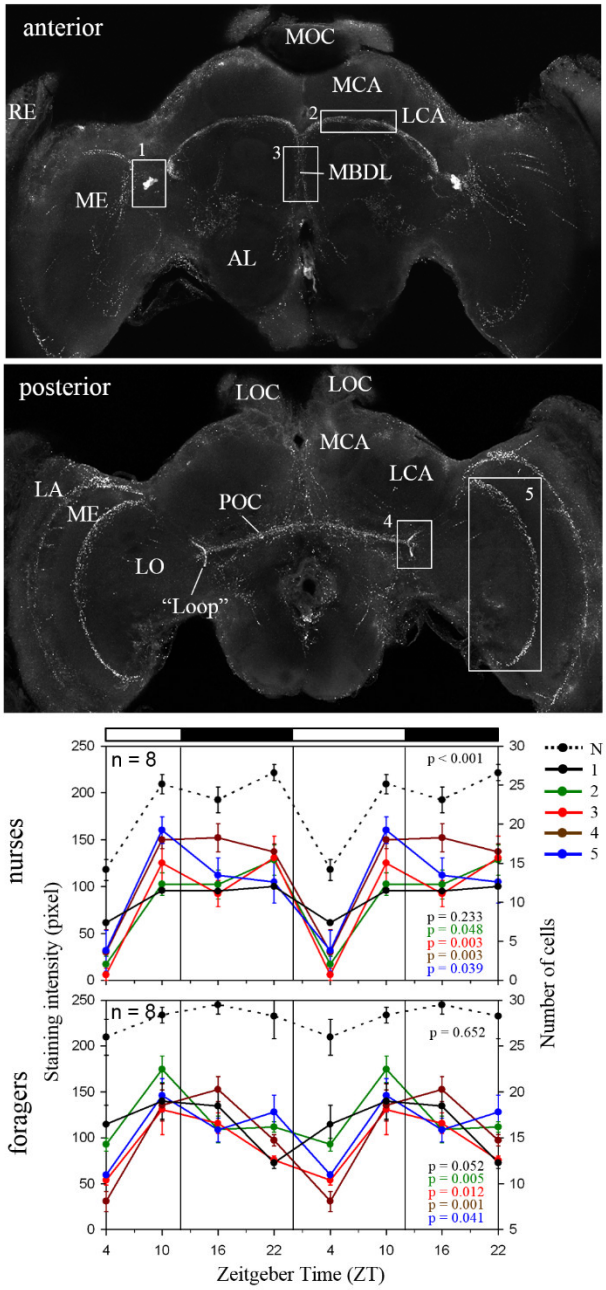

Figure 8

Figure 8. PDF immunostaining intensity over the day in nurses and foragers. The top two panels show overlays of confocal images in the anterior and posterior honey bee brain. The numbered boxes demarcate the brain areas in which we measured PDF staining intensity (rectangles 1-5). RE retina, LA lamina, ME medulla, MOC, LOC medial and lateral ocelli, MCA, LCA medial and lateral Calyx of the mushroom body. AL antennal lobe, POC posterior optic commissure, MBDL median bundle. The two lower plots summarises our records of PDF staining intensity for nurses and foragers sampled every 6 hours from an observation hive. Different colours correspond to different brain areas as shown in the upper panels and in the legend to the right: N- number of PDF-positive somata, 1 - staining intensity of the somata, 2 - staining intensity of the fibres between MCA and VL, 3 - staining intensity in the MDBL, 4 - staining intensity in the loop of the LVT, 5 - staining intensity in the posterior serpentine layer of the ME. The bars on top show the LD illumination regime, with white bars indicating light and black bars indicating darkness. For clarity the results are double plotted. Immunostaining intensity is indicated on the left y-axis and the relevant curves are shown as continuous lines in the diagrams. The

number of stained PDF neurons is indicated on the right axis and shown as stippled black line in both diagrams. Summary of the Time effect in a two-way analyses of variance (ANOVA) is shown in the bottom-right corner of each plot. The colours of the p-values correspond to the legend. Numbers of evaluated animals are given in the diagrams.

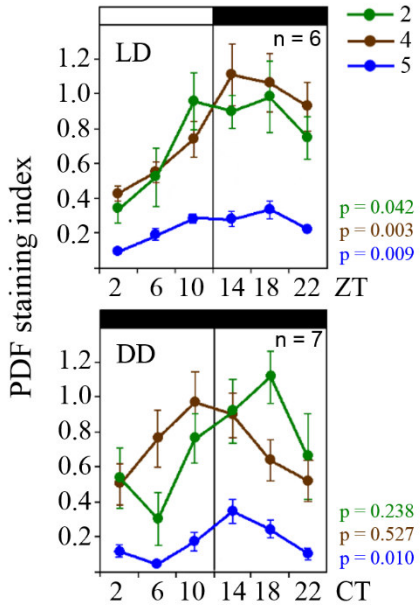

Figure 9

Figure 9. PDF immunostaining intensity over the day in foragers sampled in LD and DD illumination regimes. PDF staining intensity was evaluated at 6 different times in LD 12:12 (upper panel; n = 6 bees/ time point) and on the fourth day in constant darkness (DD; lower panel, n=7). Note that the y-axis depict the PDF staining index, the scale of which is different from the staining intensity in Figure 8. This is because the PDF staining intensity was evaluated by a different method (Hermann-Luibl et al., 2014); see Material and Methods). ZT: zeitgeber time, CT: circadian time. Other details are similar to Fig. 8.

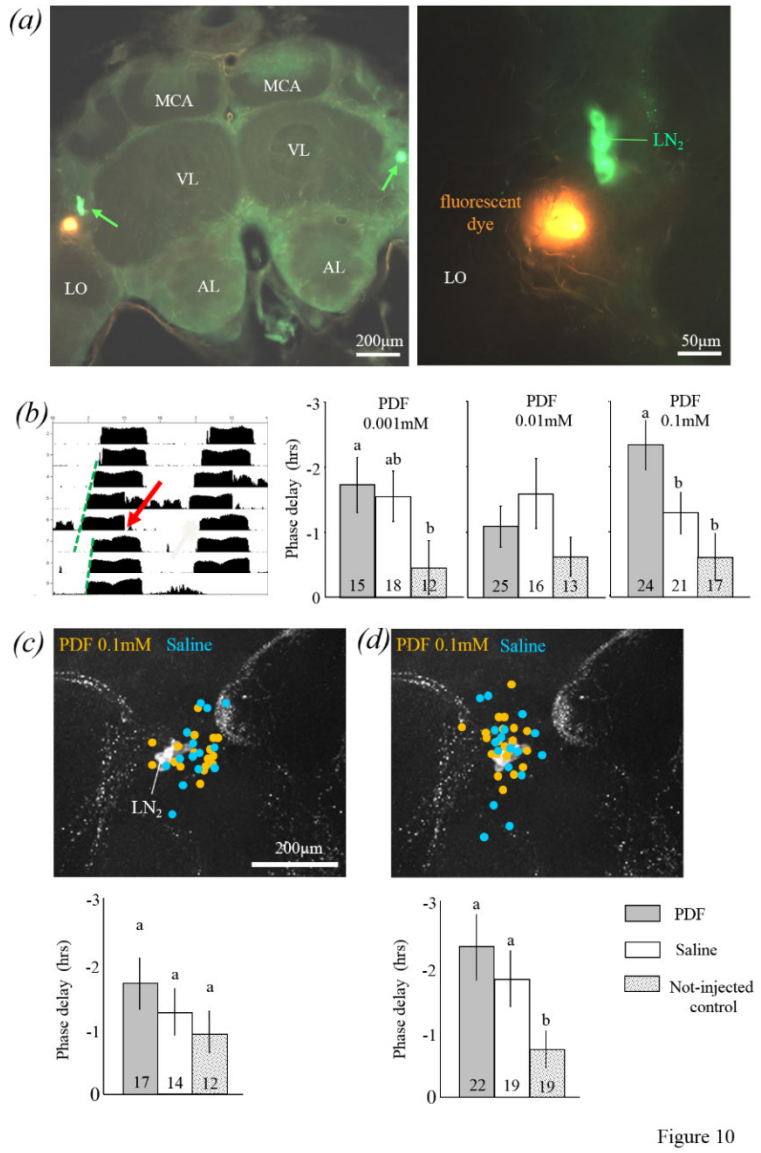

Figure 10

Figure 10. PDF and saline injection into the brain close to the PDF neurons phase delays circadian rhythms in locomotor activity. *(a)* The site of injection relative to PDF neurons (=LN_2; green arrow in left picture). The left picture gives an overview on the honey bee brain unilaterally injected with fluorescent dye (orange) and immunostained for PDF (green). The right picture is a close-up view showing that the injected die (orange) is found near LN_2 somata, but does not appear to harm them. AL antennal lobe; MCA medial calyx of the mushroom body; LO lobula; VL vertical lobe of the mushroom body. *(b)* The left picture shows a representative actogram of a honey bee forager injected with 0.1 mM PDF peptide. The red arrow points to the time of injection. The green dashed lines represent the calculated linear regression lines through the daily onsets of locomotor activity before and after the time of injections. The bee phase delays its activity onset upon PDF injection. The right picture shows the phase delays (± SD) obtained by PDF injections of three different doses. Every column depicts the pooled data of three different trials (sample size inside the bars). Only a dose of 0.1 mM PDF gave a response that was significantly different from the two controls (bars with different letters within the same plot differ statistically; two-way ANOVA, LSD post-hoc test, $p<0.05$). *(c)* Injection site and phase delays found in the first experiment, in which the injections were done through the cuticle without opening the head capsule. The injection sites were verified after the experiment and are shown as yellow and blue dots on a PDF stained brain. In this experiment, most injection sites were between the LN_2 and the superior lateral protocerebrum (SLP). The PDF injections provoked non-significant delays. *(d)* Injection site and phase delays found in the second experiment using the same method. This time, most injection sites were very close to the LN_2 (and the LN_1 that lie just dorsally of the LN2, see Fig. 3). The injections provoked large phase delays without significant differences between PDF and saline.

6.5. Buffers and Media

Buffer/Media	Ingredients/Source
Standard *Drosophila* rearing medium	0.8% agar, 2.2% sugar-beet syrup, 8.0% malt extract, 1.8% yeast, 1.0% soy flour, 8.0% corn flour, 0.3% hydroxybenzoic acid, H_2O
Immunocytochemistry	
phosphate buffered saline (PBS)	1x, (SIGMA-Aldrich (10x)), pH 7.4
phosphate buffered saline, TritonX-100 0.5% (PBST 0.5%)	1x, (SIGMA-Aldrich (10x)), 0.5% TritonX-100 (Carl Roth), pH 7.4
Sodium-Azide (NaN3)	0.02% in 1x PBS (2% stock solution, SIGMA-Aldrich)
Paraformaldehyde (PFA 4%)	4% PFA in 0.1M PBST (0.1% TrX-100)
Blocking Solution	5.0% Normal Goat Serum (NGS) in PBST 0.5% (SIGMA-Aldrich)
Zamboni's Fixative	4% Paraformaldehyde, 7,5 % saturated Picric acid solution in 0,1 M PBS, pH 7,4 (Stefanini et al., 1967)
Mounting medium for fluorescence	Vectashield ®, Vector laboratories, Ca, USA
***In vitro* larvae rearing**	
MBC(Methylbenzethonium chloride) glycerol solution	15.5 % glycerol in MBC-solution aq. (0.4 %) (SIGMA-Aldrich)
Royal jelly	Heinrich Holtermann KG, 27386 Brockel
Diet A	6% fructose, 6% glucose, 1% yeast extract, 37% H_2O, 50% royal jelly (w/w)
Diet B	7.5% fructose, 7.5% glucose, 1.5% yeast extract, 33.5% H_2O, 50% royal jelly (w/w)
Diet C	9% fructose, 9% glucose, 2% yeast extract, 30% H_2O, 50% royal jelly (w/w)

6.6. Additional material

Material	Producer/Source
Microscope cover glass (24x24 mm, 24x50 mm, 24x32 mm)	Menzel, Braunschweig, Germany
Microscope slide	Super frost Plus, Thermo Scientific, Braunschweig, Germany
Fixo GUM, Rubber cement	Marabu GmbH & Co. KG (Tamm, Germany)
Cell culture plates (48-well) with lid, sterile	Greiner bio-one, Frickenhausen, Germany
Cell culture plates (96-well) (screenstar black, TC)	Greiner bio-one, Frickenhausen, Germany
Larva grafting tool Bieno® (swiss style)	Imkereibedarf Holtermann KG, Brockel, Germany
Comb cage	Custom-made by workshop of the Biocenter, University of Würzburg
Bee aspirator	Adapted vacuum cleaner (cleanmaxx, Gallin, Germany), workshop of the Biocenter, University of Würzburg
Micro centrifuge tubes	Eppendorf, Hamburg, Germany
Micropipettes and tips	Gilson Inc., Middleton, USA
Binocular microscope (StemiSV6)	Carl Zeiss AG, Oberkochen, Germany
Forceps Dumont No. 5	SIGMA-Aldrich
featherweight forceps (for bee handling)	Carl Roth GmbH+CoKG, Karlsruhe, Germany

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List of figures and tables

Abbreviations

AME: accessory medulla

AL: antennal lobe

ALO: anteriolobular PDF hub

AOT: anterior optic tract

AOTU: anterior optic tubercle

°C: degree Celsius

CBL: lower division of the central body

CBU: upper division of the central body

CLK: clock protein

CRY: cryptochrome protein

CX: central complex

CYC: cycle protein

DAPI: 4', 6-diamidino-2-phenylindole

d: days

DD: constant conditions (darkness)

∆: delta/difference

ø: diameter

DLN: dorsolateral neurons

DN: dorsal neurons

DNA: deoxyribonucleic acid

DRA: dorsal rim area of the lamina

e.g.: for example

et al.: et alii (and others)

ES: esophageal foramen

FRP: free running period (= endogenous period τ)

h: hour

HRP: horseradish peroxidase

i.e.: id est (meaning)

IR: infrared

ITP: ion transport peptide

JH: juvenile hormone

L1-5: larval instar 1-5

LA: lamina

LAL: lateral accessory lobe

LCA: lateral lobe of the calyx

LD: light/dark

LN: lateral neurons

LO: lobula

LOC: lateral ocelli

LVT: lobula valley tract

MAL: medial accessory lobe

MBDL: median bundle

MBU: medial bulbs

MCA: medial lobe of the calyx

ME: medulla

- dME: distal medulla
- pME: proximal medulla

MEDRA: dorsal rim area oft he medulla

µm: micrometer

µl: microliter

ml: milliliter

min: minute

MOC: median ocellus

NaN_3: sodium azide

NGS: normal goat serum

NO: noduli

OC: ocelli

OL: optic lobe

P1-9: pupal instar 1-9

PB: protocerebral bridge

PBS: phosphate buffered saline

PBST: PBS with Triton-X 100

PC: protocerebrum

PDF: pigment-dispersing factor

PDH: pigment-dispersing hormone

PDP1: PAR-domain protein 1

PED: peduncle

PER: period protein

PFA: paraformaldehyde

POC: posterior optic commissure

POTU: posterior optic tubercle

qPCR: quantitative polymerase chain reaction

RE: retina

RNAi: RNA (ribonucleic acid) interference

s: second

SCN: suprachiasmatic nucleus

SEZ: subesophageal zone

SNP: superior neuropils

TIM1: timeless protein

TIM2: timeout protein

TTFL: transcriptional translational feedback loop

VL: vertical lobe of the calyx

VLNP: ventrolateral neuropils

VRI: vrille protein

ZT: Zeitgeber time

Acknowledgements

Acknowledgements

Curriculum Vitae

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* Equal contribution

Affidavit

Affidavit

I hereby confirm that my thesis entitled "**A Comparison of the circadian clock of highly social bees (*Apis mellifera*) and solitary bees (*Osmia spec.*):** Circadian clock development, behavioral rhythms and neuroanatomical characterization of two central clock components (PER and PDF)" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg, 14.11.2017

Place, Date Signature

Eidestattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „**Ein Vergleich der Inneren Uhr von sozialen Bienen (*Apis mellifera*) und solitären Bienen (*Osmia spec.*):** Entwicklung der circadianen Uhr, Verhaltensrhythmen und neuroanatomische Beschreibung von zwei zentralen Uhr Komponenten (PER und PDF)" eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

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