Circadian control of a sex-specific behaviour in Drosophila

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ABSTRACT

An endogenous circadian clock controls many of the behavioral traits of *Drosophila melanogaster*. This ¨clock¨ relies on the activity of interconnected clusters of neurons that harbor the clock machinery. The hierarchy among clusters involved in the control of rest-activity cycles has been extensively studied. Sexually dimorphic behaviors, on the other hand, have received less attention. Even though egg-laying, a female characteristic behavior, has been shown to be rhythmic, it remains largely unexplored possibly due to metholodological constraints. The current study provides the first steps towards determining the neural substrates underlying the circadian control of egg-laying. We show that, whereas the lateral ventral neurons (LNvs) and the dorsal neurons (DNs) are dispensable, the lateral dorsal neurons (LNds) are necessary for rhythmic egg-laying. Systematically probing the *Drosophila* connectome for contacts between circadian clusters and oviposition-related neurons, we found no evidence of direct connections between LNvs or DNs and neurons recruited during oviposition. Conversely, we did find bidirectional connections between Cryptochrome (Cry)

expressing LNd (Cry+ LNds) and oviposition related neurons. Taken together, these results reveal that Cry+ LNd neurons have a leading role in the control of the egg-laying rhythm in *Drosophila* females.

INTRODUCTION

Most organisms are capable of coordinating their physiology and behavior with the 24 hours of day/night cycling generated by the Earth´s rotation. These biological rhythms are driven by molecular clocks that are conserved across animals (Allada et al., 2001). In *Drosophila melanogaster* the core clock components comprise a transcription/translation feedback loop with four core proteins: CLOCK (CLK) CYCLE (CYC), PERIOD (PER), and TIMELESS (TIM) (Hardin, 2005; Tataroglu and Emery, 2015). In brief, CLK and CYC activate transcription of *period* and *timeless* genes, which, once translated into PER and TIM proteins, dimerize and translocate into the nucleus where they bind to CLK and CYC, thereby inhibiting their own transcription. This molecular feedback loop has a period of approximately 24 hours. In the *Drosophila* brain this molecular circadian clock is expressed in ~150 neurons, which are organized in different clusters based in gene expression, anatomy and localization (Helfrich-Förster, 2005; Yoshii et al., 2012). These clusters are: ventrolateral neurons (LNv; encompassing the small and large LNv groups), dorsolateral neurons (LNd), lateral posterior (LPN), and dorsal neurons [DN; separated in DN1, 2, and 3 and further subdivided into anterior (DN1a) and posterior (DN1p) DN1]. Rhythmic locomotor behavior depends primarily on the activity of the sLNvs, LNds, and DN1s, but all clusters contribute to different extents (Helfrich-Förster, 2005). Within the lateral neuron group, the sLNv are important because they drive locomotor rhythmicity under free-running (DD) conditions (Grima et al., 2004; Stoleru et al., 2004), through the release of Pigment Dispersing Factor (PDF), a neuropeptide relevant for communication between clock neurons (Renn et al., 1999; Yoshii et al., 2009).

Egg-laying is one of the most important female behaviors since it has a profound impact on the fitness of a species. Egg laying is largely governed by successful mating, but is also influenced by circadian and seasonal rhythms and by environmental factors such as temperature and food

availability, among others (Cury et al., 2019). The circadian rhythm of oviposition is one of the less studied rhythms in *Drosophila*, possibly due to the challenges involved in monitoring and recording this behavior. The periodic deposition of eggs involves a series of events ranging from the production of oocytes to the choice of the most appropriate substrate for the eggs (Allemand, 1976; Yang et al., 2008). The circadian nature of this behavior was revealed by its persistence under DD with a period around 24 h and a peak near night onset (Manjunatha et al., 2008). Egg-laying rhythmicity is temperature-compensated and remains invariant despite the nutritional state (Howlader et al., 2006). Moreover, oviposition is rhythmic in virgin females as well as in mated ones suggesting that this rhythm is not regulated by the act of mating and, instead, is endogenously driven (Menon et al., 2014).

Although oviposition exhibits a circadian component, the molecular and neural substrates that govern this rhythm have not been described. Here, using an automated egg collection device developed in our lab, we examined the contribution of the molecular clock in specific neuronal clusters. By downregulating *per* in subsets of circadian neurons, we determined that even though impairing the molecular clock in the entire clock network reduced the circadian rhythm of oviposition, restricting the disruption to DN1ps or LNvs did not. Interestingly, egg-laying rhythms disappeared upon targeting a subset of LNd clocks (Cry+ LNd) through RNAi-mediated *per* downregulation, a condition where rhythmicity of locomotor activity patterns remained unaltered, suggesting a leading and very specific role of LNd clocks/neurons in the control of oviposition. Finally, the assessment of the synaptic connectivity between clock and oviposition controlling neurons ussing the Janelia hemibrain dataset revealed direct synaptic connections between this subset of LNds and oviposition neurons, consisting with, the essential role of LNds neurons in the control of this behavior.

RESULTS

Egg-laying is rhythmic when registered with an automated egg collection device

Oviposition in *Drosophila* is one of the less studied behaviors regulated by the circadian clock. This is probably due to the difficulties involved in monitoring and recording it, and the current lack of standard devices to accomplish this. Egg collection and counting is usually done manually, making the experiments particularly demanding and labor-intensive. In this approach, eggs are typically collected every 4 hours (sometimes also every 2 hours), which usually implies transferring the fly to a new vial or extracting the food with the eggs and replacing it with fresh food in the same vial (McCabe and Birley, 1998; Menon et al., 2014). Either way this implies disturbing the fly several times a day which could alter the normal rhythm of oviposition, and demands the intervention of an experimenter every 4 hours during several days. In order to avoid this, we developed an automated egg collection device where 21 flies are individually housed, and each enclosure is slowly shifted every 4 hours from one food patch to a new food patch. Once per day all food patches are collected and eggs are manually counted (see **Material and Methods** and **Figure supplement 1** for more details).

The assessment of oviposition rhythmicity is challenging because the decision of laying an egg relies on many different internal and external factors making this behavior very noisy. In a sense, the register of the eggs laid by a female as a function of time can be considered as a periodic signal corrupted by random noise. Under light-dark (LD) conditions, or in the first days of DD, it can be assumed that the periodic signal is the same for all flies, whereas the noisy component is different for each individual. In this case it is well known (see e.g. (van Drongelen, 2007)) that averaging of several “replicates” (individual flies, in our case) leads to an improvement of the signal-to-noise ratio allowing the emergence of the underlying rhythm. As this approach seems to be better suited for detecting noisy rhythms, in the rest of the paper the assessment of rhythmicity will be based in the record of eggs laid during a given period of time averaged over all females of each genotype. **Figure 1** shows that, when eggs are collected with our device, in LD, *CS* females have a peak of egg

deposition at the end of the day, as described in the literature (Bailly et al., 2023; Howlader et al., 2006; Sheeba et al., 2001) (**Figure 1A**). **Figure 1E** shows that only about 70% of individuals are rhythmic (with a period close to 24 h, **Figure 1F**) although the average egg record is strongly rhythmic with a period around 24 h (**Figure 1B**). Under DD condition, individual rhythmicity percentages are the same as in LD (**Figure 1E**) and their average record is also very rhythmic with a period of 24 h (**Figure 1D**). It is well known that in locomotor activity experiments more than 90% of the flies are rhythmic in DD, and virtually all flies are rhythmic in LD. Thus, egg-laying displays a much larger variability than locomotor activity, compounding the difficulty of observing the influence of the circadian clock on this behavior.

In order to check that our egg collection device does not in any way entrain egg laying in flies, we tested it with *per*^{S} mutants (Konopka and Benzer, 1971). These flies are known to display a short period of locomotor activity, both in males (Konopka and Benzer, 1971) and in females (McCabe and Birley, 1998). Additionally, it has been shown that oviposition in *per*^{S} flies is rhythmic, with a period of 20.6+/- 0.9h (McCabe and Birley, 1998). Our results for this mutant are in agreement with this (**Figure 1J** shows a period of the average at 18.8+/- 1.1 h), thus confirming that flies are not entrained by our egg collection device. It is important to notice that in the control strain, the individual rhythmicity was rather low (around 50% **Figure 1K**), but the average is strongly rhythmic (**Figure 1H**) thus confirming the need for an analysis method that can take into account the low signal-to-noise-ratio of this behavior.

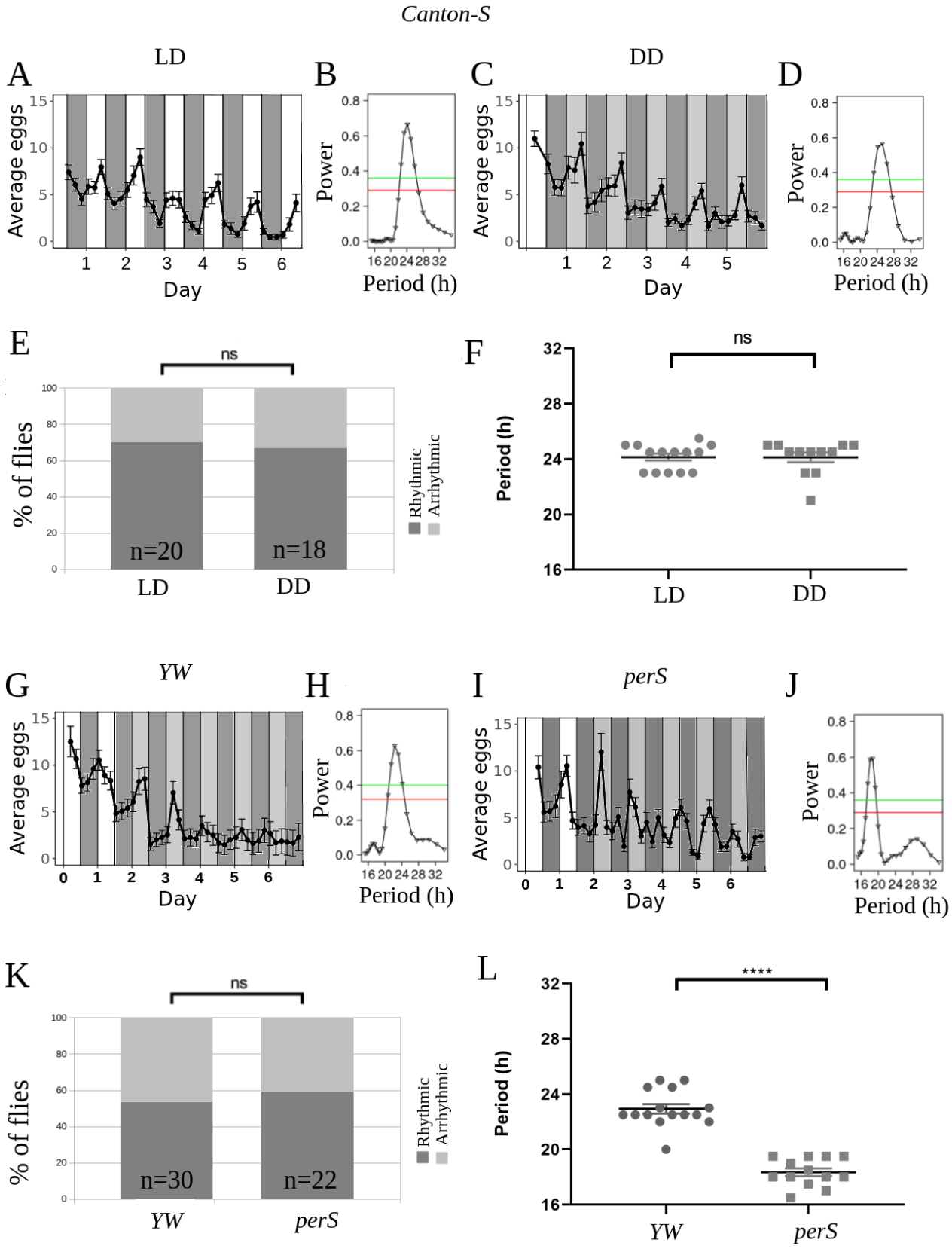

Figure 1. Oviposition in *Drosophila* is rhythmic when registered with our automated egg collection device. A, C, G, I: average eggs collected as a function of time (*Canton-S* in LD: A; *Canton-S* in DD: C; *yw*: G; *per*^{S}: I). White and dark grey bars represent periods of lights on and off respectively (LD), whereas light gray bars represent subjective days, DD, (i.e times where lights were on at rearing, but are now off). **B, D, H, J**: their respective Lomb-Scargle periodograms (*Canton-S* in LD (period 24.13 h ± 1.93 h): B; *Canton-S* in DD (period 25.21 h ± 2.17 h): D; *yw* (period 22.30 h ± 1.71 h): H; *per*^{S} (period 18.70 h ± 1.63 h): J). Red and green horizontal lines represent significances of 0.05 and 0.01,

respectively. **E, K**: Percentage of females with rhythmic oviposition (E: *Canton-S* in LD and DD, K: *yw* and per^{S}). **F, L**: Period of oviposition rhythms for individual flies (F: *Canton-S* in LD and DD, J: *yw* and per^{S}). ns: non significant, ***p<0.001 (chi-squared test).

Downregulation of *per* in clock neurons causes loss of egg-laying rhythmicity

The circadian system of *Drosophila* comprises a central clock located in the brain that controls locomotor behavior, as well as peripheral clocks located in many tissues, that regulate distinct behaviors (Franco et al., 2018; Ito and Tomioka, 2016). For example, olfaction rhythms are controlled by peripheral clocks in the antennae, and eclosion rhythms are partially controlled by peripheral clocks in the prothoracic gland (Myers et al., 2003; Tanoue et al., 2004). Earlier work proposed that oviposition might also be controlled by a peripheral clock (Manjunatha et al., 2008). In order to test for this possibility, we used RNA interference (RNAi) to knock down the clock protein PER (Herrero et al., 2017; Zhang et al., 2021) in all clock neurons in the brain (using the *Clk*856 driver (Gummadova et al., 2009)), thereby disrupting their molecular clock. First, we tested the effectiveness of this tool by monitoring the locomotor activity of males with their endogenous clock silenced in all clock neurons (*Clk*856-Gal4>UAS-per^{RNAi}). **Supplementary Table 1** shows that these flies exhibited a significant decrease in locomotor rhythmicity compared to their genetic controls. Having confirmed the effectiveness of the RNA interference, we then monitored egg-laying in females from the previously mentioned lines.

As shown in **Figure 2B**, when PER is knocked down in all clock neurons oviposition rhythmicity is drastically reduced compared to controls. The fact that knocked down animals are not completely arrhythmic could be accounted for either by the contribution of some peripheral oscillator/s relevant for the control of this behavior, or by the presence of some residual PER in clock cells (Herrero et al., 2017).

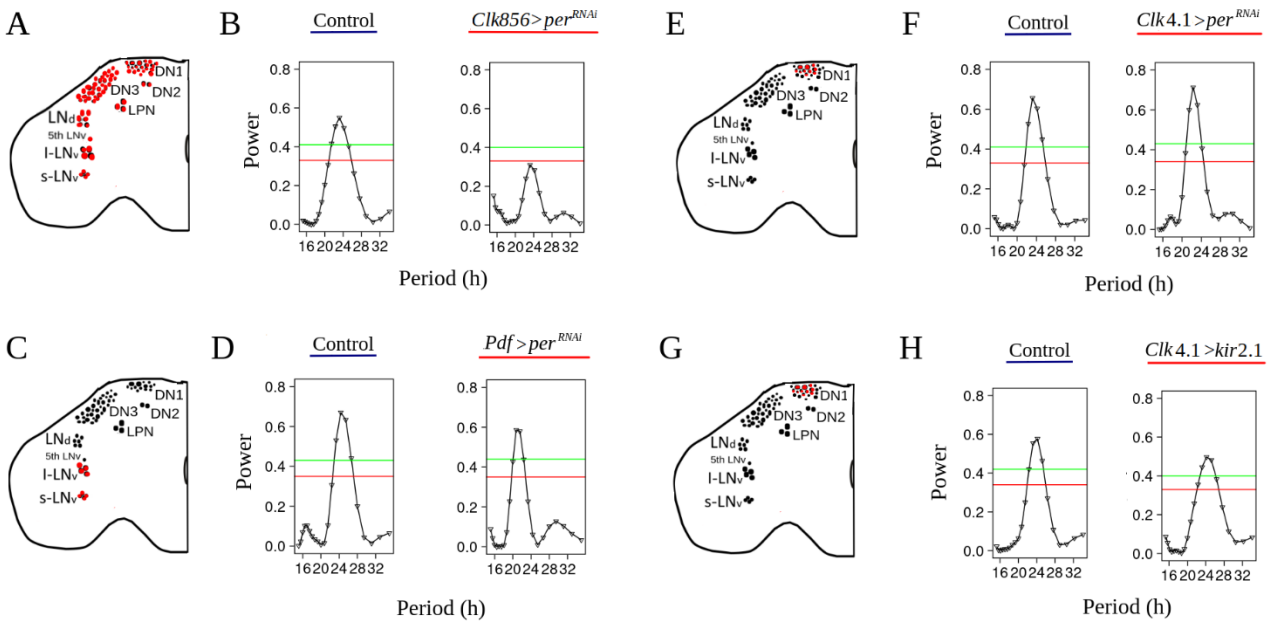

Figure 2. Circadian rhythmicity of oviposition is abolished when the molecular clock is disrupted in all clock neurons, but not when only LNv or DN1 neurons are affected. A, C, E, G: Schematic diagram of the neurons (painted in red) where the molecular clock has been disrupted. **B, D, F, G**: Periodograms of the time series of the average number of eggs laid. Red and green horizontal lines represent significances of 0.05 and 0.01, respectively. **A, B**: *Clk*856>*per*^{RNAi} (*Clk*856-Gal4>UAS-*per*^{RNAi}, n=26), Control (+>UAS-*per*^{RNAi}, period: 23.20 h ± 2.71 h n=22). **C, D**: *Pdf*>*per*^{RNAi} (*PdfDicer*-Gal4>UAS-*per*^{RNAi}, period: 20.74 h ± 1.21 h, n=34), control (+>UAS-*per*^{RNAi}, period: 24.34 h ± 1.83 h, n=18). **E, F**: *Clk*4.1>*per*^{RNAi} (*Clk*4.1-Gal4>UAS-*per*^{RNAi}, period: 22.30 h ± 1.68 h, n=40), control (+>UAS-*per*^{RNAi}, period: 23.20 h ± 1.69 h, n=38). **G, H**: *Clk*4.1>*kir*2.1 (*Clk*4.1-Gal4>UAS-*kir*2.1, period: 24.16 h ± 2.76 h, n=35), control (+>UAS-*kir*2.1, period: 24.16 h ± 2.09 h, n=36).

LNv and DN1 neurons are not necessary for egg-laying rhythmicity

We next sought to establish the role of the most important circadian groups in the control of oviposition. We concentrated on three groups: LNv, DN1p and LNd neurons. The LNvs are known to be essential for the maintenance of circadian locomotor activity rhythms, and are the only ones that express the PDF neuropeptide (Renn et al., 1999; Yoshii et al., 2009). However, we found that *per*^{RNAi} mediated disruption of the molecular clock in PDF+ cells does not abolish the time-of-day dependent oviposition (see **Figure 2D**). This is in line with previous results describing rhythmic oviposition after ablation or electrically silencing all PDF+ neurons (Howlader et al., 2006). In addition, we observed a shortening of the egg-laying period when compared to controls, (20.74 h +/- 1.21 h vs 24.35 h+/- 1.83 h respectively, **Figure 2D**), which suggests that these neurons may have some minor influence in the control of this behavior.

The DN1 neurons are not essential for the maintenance of locomotor rhythms in DD, but they contribute to the siesta (Guo et al., 2016) and generation of evening activity (Helfrich-Förster et al., 2007; Zhang et al., 2010) when the peak of egg deposition occurs. Interestingly, it has recently been shown that a posterior subset (DN1p) drives the circadian rhythm of oogenesis through the neuropeptide allatostatin C (Zhang et al., 2021). To examine whether the molecular clock of DN1p neurons is involved in the control of rhythmic oviposition, we evaluated the impact of PER downregulation on mated females expressing the *Clk*4.1 driver (which is expressed in ~10 DN1ps per hemisphere (Zhang et al., 2010)). As **Figure 2F** shows, the disruption of the clock in these neurons does not alter the rhythmicity of egg-laying in DD. To further confirm these results, and in the process examine if some other features of the DN1p could contribute to the control of oviposition, we silenced these neurons through the expression of the inward rectifier potassium channel *kir*2.1 (Baines et al., 2001). Silencing DN1p neurons did not affect the rhythmicity of egg-laying in females which showed similar patterns to those exhibited by controls females (**Figures 2H**). Taken together, these results show that neither LNv nor DN1p neurons play an important role for rhythmic oviposition.

The molecular clock in Cry+ LNd neurons is necessary for rhythmic egg-laying

The LNd neurons include 6 cells whose expression pattern is very heterogeneous (Hermann-Luibl and Helfrich-Förster, 2015; Ma et al., 2021). This group contains a subset of 3 Cry+ LNd neurons that express both *cry* (which encodes the light sensing protein cryptochrome) and *pdfr* (the PDF receptor), and 3 Cry- LNd neurons, that express neither of these genes (Im et al., 2011; Yoshii et al., 2008). Even though the LNds are not essential for rhythmic locomotor activity under constant conditions the Cry+ LNds, together with the 5-th sLNv, termed the "E oscillator", are the main drivers of evening activity (Stoleru et al., 2004). These two LNd groups greatly differ in their connectivity to the rest of the brain, since Cry+ LNds have both many more outputs and inputs than the Cry- LNds (Shafer et al., 2022). In order to assess the role of the Cry+ LNds in the control of oviposition, we

resorted to the very specific MB122B driver (Duhart et al., 2020; Guo et al., 2017) to perform an RNAi-mediated disruption of the molecular clock in those neurons. As **Figure 3B** shows, impairing clock function in Cry+ LNds results in a drastic reduction of rhythmic egg-laying. To further confirm this observation, we employed a different Gal4 driver to direct expression to E cells. *Mai*179-Gal4;*pdf*-Gal80 (Grima et al., 2004; Picot et al., 2007) is expressed in the 5th sLNv, 3 Cry+ LNds, and also drives weak expression in a small subset of DN1s. As expected, under these conditions rhythmic egg laying was severely compromised (**Figure 3D**), providing further confirmation that the molecular clock in Cry+ LNd neurons is necessary for the generation of egg-laying rhythms.

It has recently been shown that disrupting the molecular clock in E cells by means of the MB122B driver did not alter locomotor activity rhythms (Bulthuis et al., 2019). This would imply that locomotor activity and oviposition rhythms are controlled by distinct groups of clock neurons. However, Bulthuis et al. (2019) only assessed the locomotor activity of male flies, and it is well known that, unlike males, females undergo important changes in their activity after mating, such as the loss of the midday siesta (Elwyn Isaac et al., 2010) or the morning anticipation (Riva et al., 2022). For these reasons, we decided to examine the influence of the molecular clock of E cells directly on the locomotor activity of mated females. The standard setup for assessing locomotor activity is the Drosophila Activity Monitoring (DAM) system (Pfeiffenberger et al., 2010), where flies are housed in small glass tubes (of 5mm in diameter). Such arena does not offer an adequate environment for testing flies that lay eggs during several days; instead, we monitored activity using an alternative system developed in our lab, where flies are housed in much larger cubicles and their activity is recorded by a video system (see Methods and (Riva et al., 2022)). Interestingly, mated females with disrupted E cell clocks were as rhythmic as control flies (**Figure 4**), showing that there is no sex-specific contribution of this cluster to the control of rhythmic rest-activity cycles, which opens the possibility that E cells differently contribute to the control of oviposition and locomotor activity. In other words, our results show that these two important rhythmic behaviors are primarily controlled by distinct groups of clock neurons.

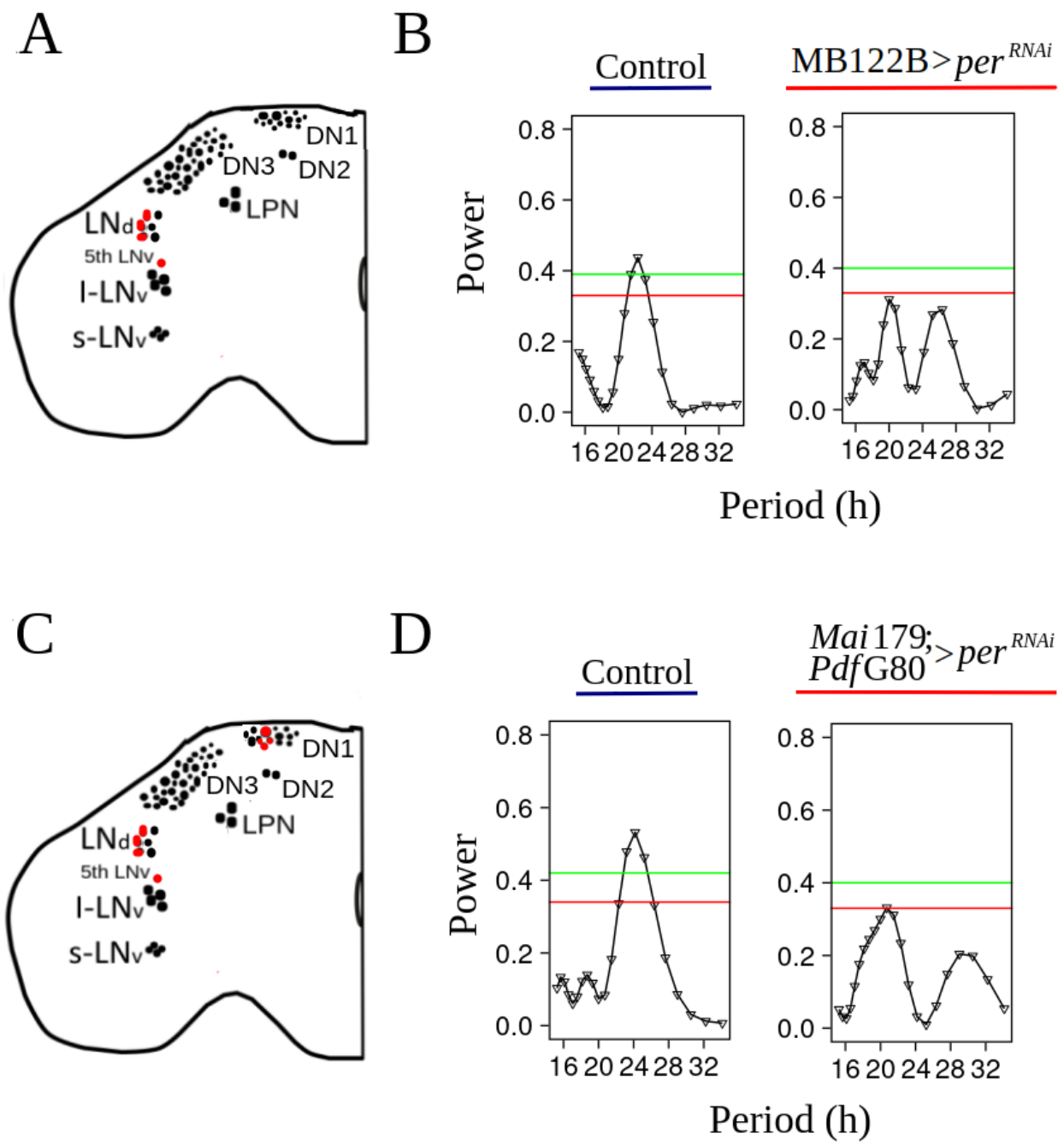

Figure 3. Disruption of the molecular clock in Cry+ LNd neurons drastically reduces the circadian rhythmicity of oviposition. A, C: Schematic diagram of the neurons (painted in red) where the molecular clocks have been disrupted. **B, D**: Periodograms of the time series of the total number of eggs laid by females. Red and green horizontal lines represent significances of 0.05 and 0.01, respectively. A, B: MB122B>*per*^{RNAi} (MB122B-splitGal4>UAS-*per*^{RNAi}, n=21), control (+>UAS-*per*^{RNAi}, period: 22.30 h ± 1.93 h, n=25). C, D: *Mai*179; *Pdf*G80>*per*^{RNAi} (*Mai*179-Gal4; *pdf*-Gal80>UAS-*per*^{RNAi}, n=19), control (+>UAS-*per*^{RNAi}, period: 24.16 h ± 2.24 h, n=14).

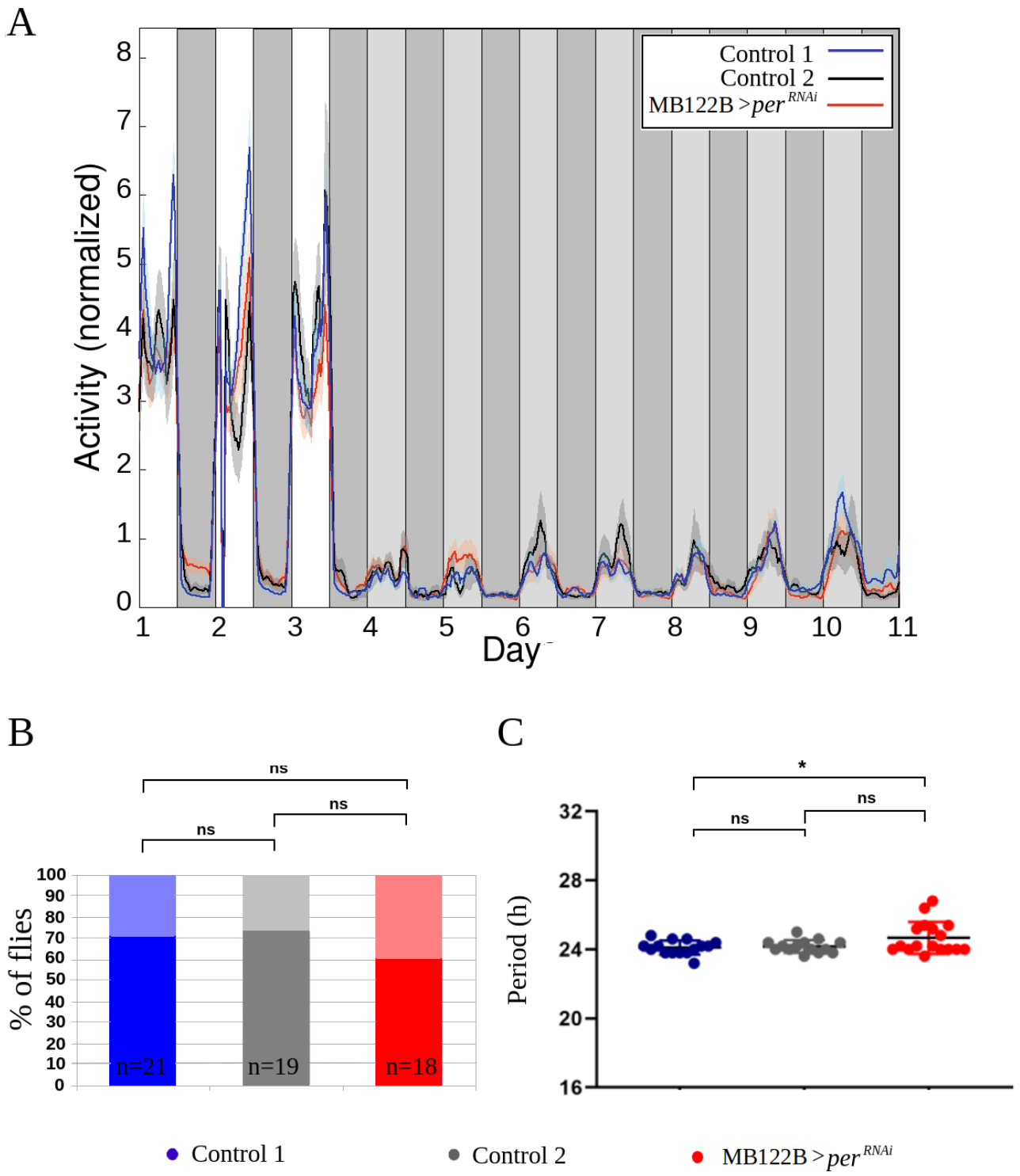

Figure 4. Disruption of the molecular clock in Cry+ LNd neurons does not alter the circadian rhythmicity of locomotor activity of mated female flies. A: Average activity recording during 3 days in LD and 7 in DD, shading represents average + SEM. Light grey bars represent subjective days. **B**: Percent of rhythmic flies in DD. **C**: Periods of locomotor activity in DD of individual flies. Mean ± SEM indicated. Each dot represents one fly. Genotypes analyzed: MB122B>*per*^{RNAi} (MB122B-splitGal4>UAS-*per*^{RNAi}), control1 (+>UAS-*per*^{RNAi}), control2 (MB122B-splitGal4>+). ns: non significant, *: $p<0.05$ (one-way-ANOVA with post-hoc Tukey).

Cry+ LNd neurons directly contact oviIN

If the circadian clock influences oviposition, at least some clock neurons should be connected, directly or indirectly, to the neurons in the brain that control the motor program leading to egg deposition. These neurons, that were recently characterized (Vijayan et al., 2023a; Wang et al., 2020; Zhou et al., 2014), include: the oviposition descending neurons (oviDN, 5 per hemisphere), the oviposition excitatory neurons (oviEN, 1 per hemisphere), the oviposition inhibitory neurons (oviIN, 1 per hemisphere), and pC1 neurons (5 per hemisphere). The best tool in hand to systematically probe the connectivity between circadian clock and oviposition-related neurons is the *Drosophila* hemibrain connectome (Scheffer et al., 2020). This is the result of the FlyEM connectomics project, which used electron microscopy images of the brain of a 5-day old female fly to reconstruct the connectivity map (or connectome) of ~25000 neurons, mostly from the central part and the right lateral part of the brain.

28 of these neurons have been identified as clock neurons, and are located in the right part of the brain (except for one LPN (Scheffer et al., 2020)). These include the best characterized circadian groups (sLNvs, lLNvs, LNds). For other groups, such as the DN1s and LPNs, only a minor fraction have been identified, whereas for groups DN2 and DN3 no neurons have yet been identified in this volume.

Interestingly, almost all oviposition-related neurons have been identified in the hemibrain (only two oviDN on the left hemisphere are still missing), and very recently a couple of additional groups called U and G, comprising 2 and 5 neurons each, respectively, have also been shown to be important for oviposition and mapped to the connectome (Vijayan et al., 2023b). This makes a total of 25 oviposition-related neurons identified thus far.

To address the possibility of direct connections between clock neurons and neurons involved in egg laying, we probed the hemibrain connectome for these connections. The full results are displayed in **Supplementary Tables 2 and Table 3**, but in short, we found that whereas there are no connections between LNv or DN1 neurons and oviposition neurons, LNds contact oviIN and pC1 neurons (**Figure**

5), further supporting the experimental findings described in previous sections. Interestingly, different subgroups of E cells (Shafer et al., 2022) show distinct patterns of connectivity to oviposition neurons: E3 neurons (comprising Cry- LNd1, LNd2 and LNd3) only contact the pC1 group, while E2 neurons (comprising Cry+ LNd4 and LNd5) only contact the oviIN neurons (**Figure 5**). On the other hand, the E1 neurons (comprising Cry+ LNd6 and 5-th sLNv) make no contacts with oviposition related neurons. This evidence suggests that the subset of E cells responsible for the loss of rhythmic oviposition are likely E2 neurons.

Interestingly, we noticed that connections between clock and oviposition neurons are bidirectional, although the E2-oviIN connection is stronger in the direction from the clock to oviposition neurons. This analysis suggests that, even though some feedback is anticipated, the E2 neurons are likely the ones providing temporal information to the oviposition circuit.

Additionally, aside from the circadian groups considered so far, we found strong, bidirectional connections between the clock neurons LPN (Reinhard et al., 2022) and the oviIN, with the strongest connections going from oviIN to LPN (**Supplementary Tables 2 and 3 and Figure supplement 2**).

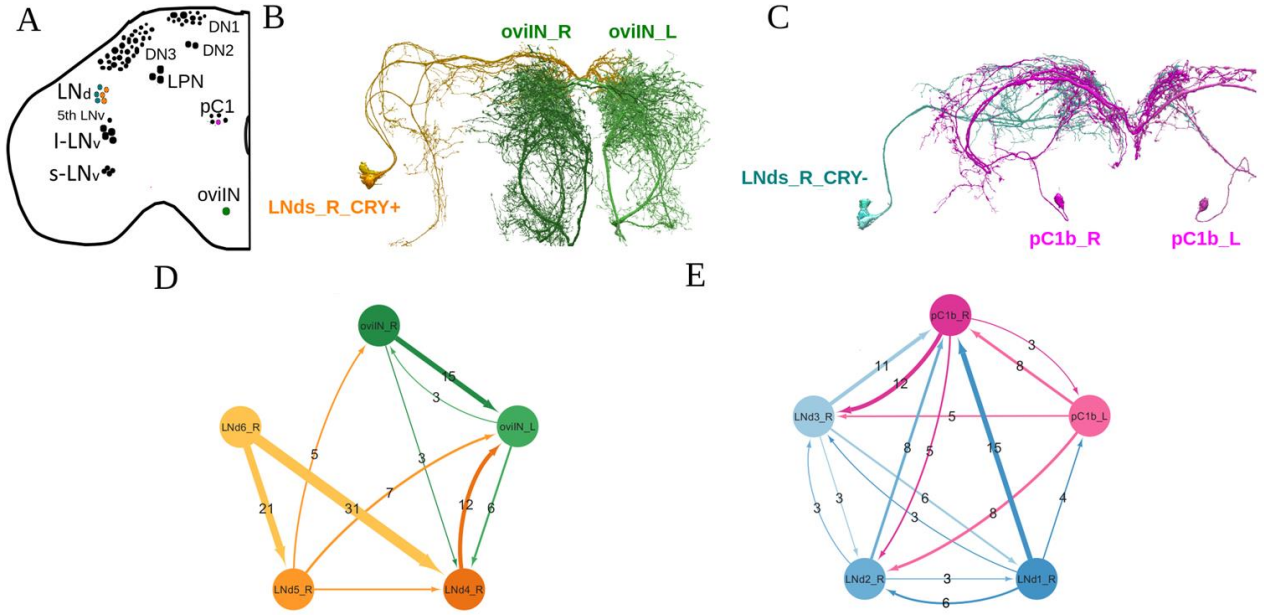

Figure 5. Direct synaptic connections between circadian clock neurons and oviposition-related neurons in the hemibrain dataset. A: Schematic diagrams showing the different neuron clusters analyzed (in color). **B**: Connection between Cry+ LNd and oviIN neurons. **C**: Connection between Cry- LNd 2and pC1b neurons. **D**: Network representation of the connectivity between Cry+ LNd and oviIN neurons. **E**: Network representation of the connectivity between Cry- LNd and pC1b neurons. Numbers give the number of synaptic contacts, which represent the strength of the connections. Only intermediate (between 3 and 9 synaptic contacts) and strong (>9 synaptic contacts) connections are considered.

DISCUSSION

The oviposition rhythm is one of the less studied behavioral traits of *Drosophila*. One possible reason is merely technical, since egg collection and counting are a very laborious processes and there are no standard devices (just as the DAM system for locomotor activity) to reproducibly evaluate it. Data analysis and rhythmicity assessments are also particularly difficult because the presence or absence of a rhythm must be established with only approximately 6 data points per cycle (in contrast to locomotor activity time series, where hundreds of data points per cycle are obtained), which can take only a few discrete values (typically, from 0 to 10). The analysis is further complicated by the fact that the daily average of eggs laid decreases with time (Kaufman and Demerec, 1942). To tackle these limitations, we developed an automated method for egg collection and a new pipeline for data analysis. We observed that the oviposition rhythm is less consolidated that locomotor activity rhythms. Surprisingly, this happens even in LD, where time of day cues are provided by light, the

most important *zeitgeber* for diurnal animals. Egg-laying is even less rhythmic in DD since the information from the endogenous clock is one among many internal and external factors that influence the decision of laying an egg. In a sense, the oviposition record of a single fly can be considered as periodic signal with strong noise added to it. Such data might not even look rhythmic when analyzed individually. However, if we assume that the periodic component is the same for all flies in a population, and the noise is different for each individual, the periodic component can be extracted by averaging the oviposition data. This led us to prefer periodograms of averaged data for the assessment of the rhythmicity of a given genotype.

Even though there have been some advances in the understanding of the relationship between circadian clock and oviposition (Howlader and Sharma, 2006; Manjunatha et al., 2008), there is still no information about the relative importance of the different neuronal groups in driving this behavior. Here, we downregulated the molecular clock in subsets of circadian neurons in order to establish their role in the control of circadian oviposition. First, we found that animals with downregulated *per* expression in all clock neurons of the brain display a drastic reduction of rhythmic oviposition, underscoring a key role of the central clock (as compared with peripheral clocks) in the control of the timing of oviposition.

We also disrupted the molecular clock (or electrically silenced) in PDF-expressing neurons as well as in the DN1p group with no apparent effect on egg laying rhythms, confirming previous reports (Howlader et al., 2006). Taken together, these results show that communication between either group and any other neurons is not necessary for rhythmic oviposition. This is in stark contrast with the situation for locomotor activity, which has been shown to become arrhythmic when PDF+ cells are ablated (Grima et al., 2004; Stoleru et al., 2004). Interestingly, however, it has been recently shown that disrupting the molecular clock in these same neurons does not alter locomotor rhythmicity (Delventhal et al., 2019; Schlichting et al., 2019) suggesting a more complex scenario.

Since it has been shown that the DN1p generate the rhythm of oogenesis (Zhang et al., 2021), one might wonder why the disruption of their molecular clock or their electrical silencing does not also

abolish the rhythm of oviposition. It is important to understand that the suppression of the oogenesis rhythm does not necessarily have an impact on rhythmic egg laying. The persistence of the rhythm of oviposition implies that it is not based on the availability of eggs but is instead an intrinsic property of the motor program, which is in fact the hypothesis more favored by our results. Somewhat surprisingly, the disruption of the molecular clock in Cry + LNd neurons led to an almost complete loss of the oviposition rhythm. In contrast, downregulating the clock in Cry+ LNds did not affect rhythmic locomotor activity in mated females, underscoring a degree of specificity. Interestingly, in males the Cry+ LNds have been singled out as responsible for driving the activity peak at dusk (evening peak, (Guo et al., 2017)), which is the time when, in females, egg laying has a peak. As far as we know, oviposition is the first female-specific behaviour specifically controlled by the LNds. In males the LNds have been shown to be involved in the circadian control of the expression of sex-specific fat body genes (Erion et al., 2016; Fujii et al., 2008).

The neural circuitry that controls the egg laying process has just begun to be unraveled (Vijayan et al., 2023a; Wang et al., 2020; Zhou et al., 2014). It includes the oviDNs, oviINs, oviENs, groups U and G, and pC1 neurons. Using the hemibrain connectome we found that neither the identified DN1s nor the LNv neurons directly contact any neurons ascribed to the oviposition circuit; the LNd cluster, on the other hand, exhibits synaptic contacts with pC1 and oviIN neurons, providing the anatomical substrate for our experimental results. Furthermore, there is a clear separation of roles inside the LNd cluster, since the oviINs are only contacted by two Cry+ LNds (the so-called E2 cells (Shafer et al., 2022)) and the pC1s are only contacted by the three Cry- LNds (E3 cells (Shafer et al., 2022)). There is also a functional separation within the pC1 cluster, since the only pC1 neurons connected E3 neurons are the pC1b neurons from both right and left hemispheres (**Figure 5**). The resulting clock/oviposition network is summarized in **Figure 6**.

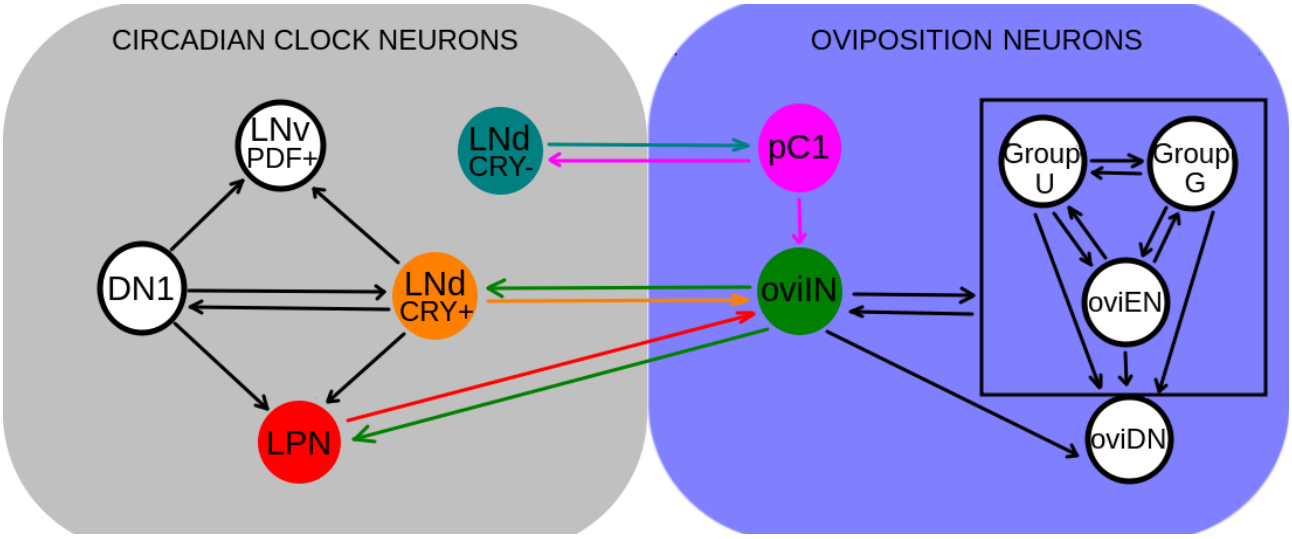

Figure 6. Scheme of connections between circadian clock and oviposition-related neuron clusters of the same hemisphere in the hemibrain dataset. Each circle represents a neuron cluster, comprising different numbers of neurons (some clusters comprise only one neuron). Clusters and connections involved in the connectivity between circadian clock and oviposition sets have been colored. In each connection the arrow points to the post-synaptic cluster. OviIN neurons are bidirectionally connected to every neuron of the clusters inside the square.

Our data posits the E2 LNds-oviIN connection as the candidate to mediate the timing of egg laying rhythms. The oviIN neurons are two of the most important neuronal hubs included in the current version of the *Drosophila* connectome, receiving strong inputs of hundreds of other neurons. Thus, the oviINs integrate the information from the circadian clock with the information coming from many other sources in order to signal to the oviposition circuit. Under such scenario the circadian clock would compete with many other sources to set the threshold for egg deposition (Vijayan et al., 2023a).

We hypothesize that the E3-pC1b connection is less important for conveying circadian information to the oviposition circuit because the pC1b are located upstream within the oviposition circuit that controls the motor program, being connected only to the oviIN neurons (Wang et al., 2020). On the other hand, since pC1 neurons control female receptivity, it is tempting to speculate that this behavior may have a circadian rhythm, that could be generated through the E3-pC1b connection. Interestingly, the female mating rate has been shown to display circadian rhythmicity (Sakai and Ishida, 2001), and very recently it has been proposed that pC1b neurons are involved in the control

of female copulation rate (Li et al., 2023). Conversely, given that the E3-pC1b connection is bidirectional, it could also be instrumental in conveying information about mating status to the circadian clock, therefore producing the changes in temporal organization that females undergo after mating (Delbare et al., 2023; Elwyn Isaac et al., 2010; Riva et al., 2022).

The function of the LPN neurons in the circadian clock is still unclear (Reinhard et al., 2022) and therefore no clear role in rhythmic oviposition can be ascribed to the LPN-oviIN connection. On the other hand, since there are many more oviIN inputs to the LPN than outputs (135 vs 16 synaptic connections), it seems more likely that this connection conveys information to the circadian clock from those processes in which the oviINs are involved.

The information provided by the *Drosophila* connectome is only the first step towards the understanding of the influence of the circadian clock on oviposition. Future work will be necessary to test the functionality of these connections as well as the role of different neuropeptides (sNPF, ITP, PDF) and neurotransmitters (such as acetylcholine and glutamate) in the control of egg laying behavior.

MATERIAL AND METHODS

Fly strains

All fly strains used in this study are detailed in Table 1. Flies were reared and maintained on standard cornmeal/agar medium at 25 °C and 60% humidity in a 12 hr:12 hr LD cycle unless stated otherwise. For oviposition and locomotor activity experiments we used a different medium, named banana medium, prepared with 200g of banana, 25g of barley, 36g of black sugar, 35 g of yeast, 12.5g of agar and 2g of Nipagin per liter of water [15].

Table 1

All fly strains used in this study.

***Drosophila melanogaster* line**	**Source**	**Identification number**	**Common denomination**
Canton-S	*Bloomington Drosophila Stock Center*	BDSC: 64349	*CS*
Yellow white	*Bloomington Drosophila Stock Center*	BDSC: 1495	*y w*
y per^{S} w;	*Donated by Jeff Hall*	Collection of Jeff Hall	*per^{s}*
y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS 02045}attP2/TM3, Sb[1]	*Bloomington Drosophila Stock Center*	BDSC:40878	UAS-*per^{RNAi}*
w[*]; P{w[+mC]=Clk-GAL4.-856}2	*Bloomington Drosophila Stock Center*	BDSC:93198	*Clk856*-Gal4
w[*]; sna[Sco]/CyO; P{w[+mC]=Clk-GAL4.1.5}4.1M/TM 6B, Tb[1]	*Bloomington Drosophila Stock Center*	BDSC:36316	*Clk*4.1-Gal4
P{w[+mC]=Pdf-	*Bloomington Drosophila Stock Center*	BDSC:6899	*PdfDicer*-Gal4

GAL4.P2.4}X, y[1] w[*];Dicer/cyo			
*Mai*179-Gal4;*Pdf*-Gal80	Donated by José Duhart	Collection of Patrick Emery	*Mai179-Gal4;Pdf-Gal80*
MB122B E-cell split-Gal4	Donated by José Duhart	Collection of Orie Shafer	*MB122B*-splitGal4
w;; kir2.1(1)/TM3 Sb	Donated by Justin Blau	Collection of Justin Blau	UAS-*kir*2.1

Automated egg collection device

The egg collection device (**Figure supplement 1**) consists of a wooden basis with a mechanical arm fixed to it, and plastic sets of tracks with fly chambers that can be replaced at will. Each "set of tracks" is a 13.9 x 19.5 cm 3D-printed piece with 7 tracks. Inside each track there are 6 equidistant wells measuring 15 x 20 mm, which are filled with banana medium. Each fly chamber (17 x 27 mm) has a transparent roof, and no floor, so that the fly inside it is in contact with the food wells, when the chamber is placed on the track so that it can slide along it. 3 sets of tracks are placed on the wooden base, and the chambers are moved together from well to well by a mechanical arm, which is moved by a stepper motor controlled by an Arduino UNO. The chambers are displaced from well to well every 4 hours. More frequent sampling gives rise to less consistent rhythmic patterns.

Behavioral assays

The egg deposition behavior of females was analyzed individually at 25°C and 60% humidity. Before starting the assay, six 0-5 day old virgin females and five males of the desired genotype were anesthetized using CO_2 and introduced into a vial with 10mL of standard food for 72 hours. This allowed for the crossing of 50-80 female flies with their respective males per experiment.

Subsequently, the resulting gravid females from the crosses were placed individually in vials containing a plastic spoon with 1.5mL banana medium along with a drop of yeast for one day. The eggs deposited by each of the females are counted under a binocular stereoscopic magnifier (Lancet Instruments) and the 21 best egg layers are selected to conduct the circadian oviposition experiment. This prior selection of the best egg layers is performed because, of the total females put to mate with males, nearly half do not lay eggs or lay very few.

21 of these females were housed in the chambers of each apparatus, which was then introduced in an incubator at constant temperature (25 °C). The first 1 or 2 days the light regime was LD (12:12) in order for the flies to adapt to their new environment, and then they entered a DD regime for a given number of days. The sets of tracks were retired and replaced by sets with new food every 20, 24 or 28 hours (in order to avoid providing an entrainment signal to the flies). The eggs in each food well of each of the retired tracks were counted under a binocular stereoscopic magnifier (Lancet Instruments), and registered.

For the locomotor activity assays we used the setup used in previous works (Riva et al., 2022). Briefly, the flies are housed in translucid tracks and their movement is registered with video cameras above the tracks, connected to a computer, where the position of each fly is extracted from the video (with ad hoc programmed software) and registered. Locomotor activity of mated females was monitored for 3 days in LD conditions and then transferred to DD conditions for 7 days. Every 4 or 5 days the flies were transferred to tracks with fresh food, because after that time the appearance of larvae hindered the video tracking of the females.

Data analysis

After each oviposition experiment lasting N days the data consisted of a time series *E* of 6N points for each fly. A new time series was generated by averaging the individual series. Since the number of eggs laid by a mated female tend to show a downward trend, we proceeded as follows, in order to detrend the data (see the Supplementary Material for further details). First, a moving average of

the data is performed, with a 6 point window, and a new time series *T* is obtained. In principle, *T* is a good approximation to the trend of the data. Then, a new, detrended, time series *D* is generated by pointwise dividing the two series (i.e. *D(i)=E(i)/T(i)*, where *i* indexes the points of each series).

In order to assess the rhythmicity of a genotype, we averaged the detrended time series and performed a Lomb-Scargle periodogram (Ruf, 1999; VanderPlas, 2018). For this we used package Lomb (version 2.1.0), from the R Statistical Software (v4.1.2; R Core Team 2021). The significance lines were calculated by repeatedly randomizing the time series (using function *randslp* in Lomb R package). A genotype was considered rhythmic if the periodogram had one peak between 16 and 32 hs, and it was above the p=0.05 significance line. The period mentioned in figure captions (Figures 1, 2 and 3) correspond to the position of the peak ± half width at half peak.

The graphs showing individual periods were made by obtaining the individual periods using the Lomb-Scargle periodogram described above for each particular individual. For the individual periods shown in the figures, we added the weakly rhythmic category, corresponding to flies whose periodograms displayed only one peak between 18 and 32 hs, and a power larger than 0.2.

For locomotor activity experiments, these files obtained from the video tracking were processed with an analysis software we developed (in Bash) which provides statistics for activity (position, distance traveled, etc.) (Riva et al., 2022).

Analysis of connectome data

The data used to determine the connectivity between the circadian clock and the oviposition circuits come from the Hemibrain dataset (version 1.2.1) made publicly available by Janelia Research Campus (Scheffer et al., 2020). To access the data we used the NeuPrintExplorer Web tool (https://neuprint.janelia.org/). The result of our queries is summarized in **Supplementary Tables 2 and Table 3**. The neuron IDs are as given in the hemibrain dataset, just as the instance names of the oviposition related neurons. For circadian clock neurons we used the names given by Shafer et al. (Shafer et al., 2022). We only replaced LPN-4 by LPN-L to stress the fact that this neuron is placed in

the left hemisphere (all the others are in the right hemisphere). The strength of each connection was quantified using the criteria of Scheffer et al. (Scheffer et al., 2020). Connections with more than 9 synapses were considered strong, connections having between 3 and 9 synapses were considered as having intermediate strength, and connections with 0,1, or 2 synapses were considered as weak. Because weak connections are prone to error (Scheffer et al., 2020) we excluded them from the analyses. The figures that show connectivity between sets of neurons were drawn using Cytoscape version 3.10.1 (Shannon et al., 2003).

Statistical analysis

The following statistical analyses were used in this study: We tested data for normality with D'Agostino & Pearson test. Once the normality of the data was confirmed, we used a unpaired t-test for test differences between means for experiments where 2 groups were compared and a one-way ANOVA for experiments where 3 groups were compared. When data was not normally distributed we applied a Mann-Whitney test for test differences between means. When significant differences were found, we conducted a post-hoc Tukey test with a correction for multiple comparisons. In all the analyses we used Two-Tailed *p*-values. To perform all the tests, we used GraphPad Prism (Boston, Massachusetts USA, www.graphpad.com) and R Statistical Software (v4.1.2; R Core Team 2021). $p < 0.05$ was considered statistically significant. In dot plots, horizontal lines represent the mean value; error bars depict the standard error of the mean. No statistical methods were used to determine sample size. Sample sizes are similar to those generally used in this field of research. Samples were not randomized and analyzers were not blind to the experimental conditions. In all the figures we show results of two or three independent experiments.

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AUTHOR CONTRIBUTIONS

Conceptualization, Methodology and Validation: S.R; S.R.G; D.L.F; Formal Analysis, Investigation, and Visualization, S.R; S.R.G; D.L.F; Writing – Original Draft, S.R; S.R.G; D.L.F; Writing – Review & Editing: S.R, M.F.C; S.R.G, D.L.F ; Supervision, S.R.G, D.L.F ; Project Administration and Funding Acquisition, M.F.C, S.R.G., D.L.F.

DECLARATION OF INTEREST

The authors declare no competing financial interests.