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* positive 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 [1]. 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) [2,3]. In brief, CLK and CYC activate transcription of *period* and *timeless* 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 [4,5]. 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 [4]. Within the lateral neuron group, the LNv are important because they drive locomotor rhythmicity under free-running (DD) conditions [6,7], through the release of Pigment Dispersing Factor (PDF), a neuropeptide relevant for communication between clock neurons [8,9].

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 [10]. 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 [11,12]. 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 [13]. Egg-laying rhythmicity is temperature-compensated and remains invariant despite the nutritional state [14]. 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 [15].

Although oviposition exhibits a circadian component, the molecular and neural substrates that govern this rhythm have not been described. Here, we examined the contribution of the molecular clock in specific neuronal clusters. We downregulated *per* in subsets of circadian neurons and monitored rhythmic egg-laying with a new device that we developed. Thus, we determined that despite 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 Cry+ LNd clocks through RNAi-mediated *per* downregulation, a condition where rhythmicity of locomotor activity patterns remained unaltered, suggesting a leading role of LNd clocks/neurons in the control of oviposition. Finally, the assessment of the synaptic connectivity between clock and oviposition controlling neurons described in the Janelia hemibrain dataset revealed direct chemical synaptic connections between the LNds and oviposition neurons, reinforcing the essential role of LNds neurons.

RESULTS

Egg-laying is rhythmic when registered with an automatic oviposition 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. Likewise, the assessment of rhythmicity is more difficult than for locomotor activity because the variable studied (number of eggs) is discrete and usually small when recorded for periods of a few hours. Even though population-level experiments can partially overcome these difficulties, social interactions can influence the oviposition behavior of *Drosophila* females [16], making the results much harder to interpret.

We developed an automatic device that allows the simultaneous monitoring of 21 flies individually housed, and an analysis method that takes into account the particularities of the data (see Material and Methods). In order to validate our setup, we compared the egg-laying rhythmicity in wild type genotypes recorded by our apparatus, which stores the eggs laid every four hours for each mated fly, with records obtained manually at the same frequency. **Figure 1** shows that, for mated *Canton-S* (*CS*) females in constant darkness, no significant differences were found in oviposition rhythmicity and period length between both systems, which validates our approach.

Figure 1 also shows that, in LD, *CS* females have a peak of egg deposition at the end of the day, as described earlier [14,16,17] (**Figure 1A and 1E**) and that rhythmicity is between 60% and 75% (**Figure 1I**). In DD, rhythmicity percentages can be even smaller. In contrast, 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 partially circumvent variability issues, we assessed rhythmicity by analyzing periodograms of the total eggs

laid by all flies (as, for example, in **Figures. 1B, F, D, H**) in every experiment (despite flies were individually housed).

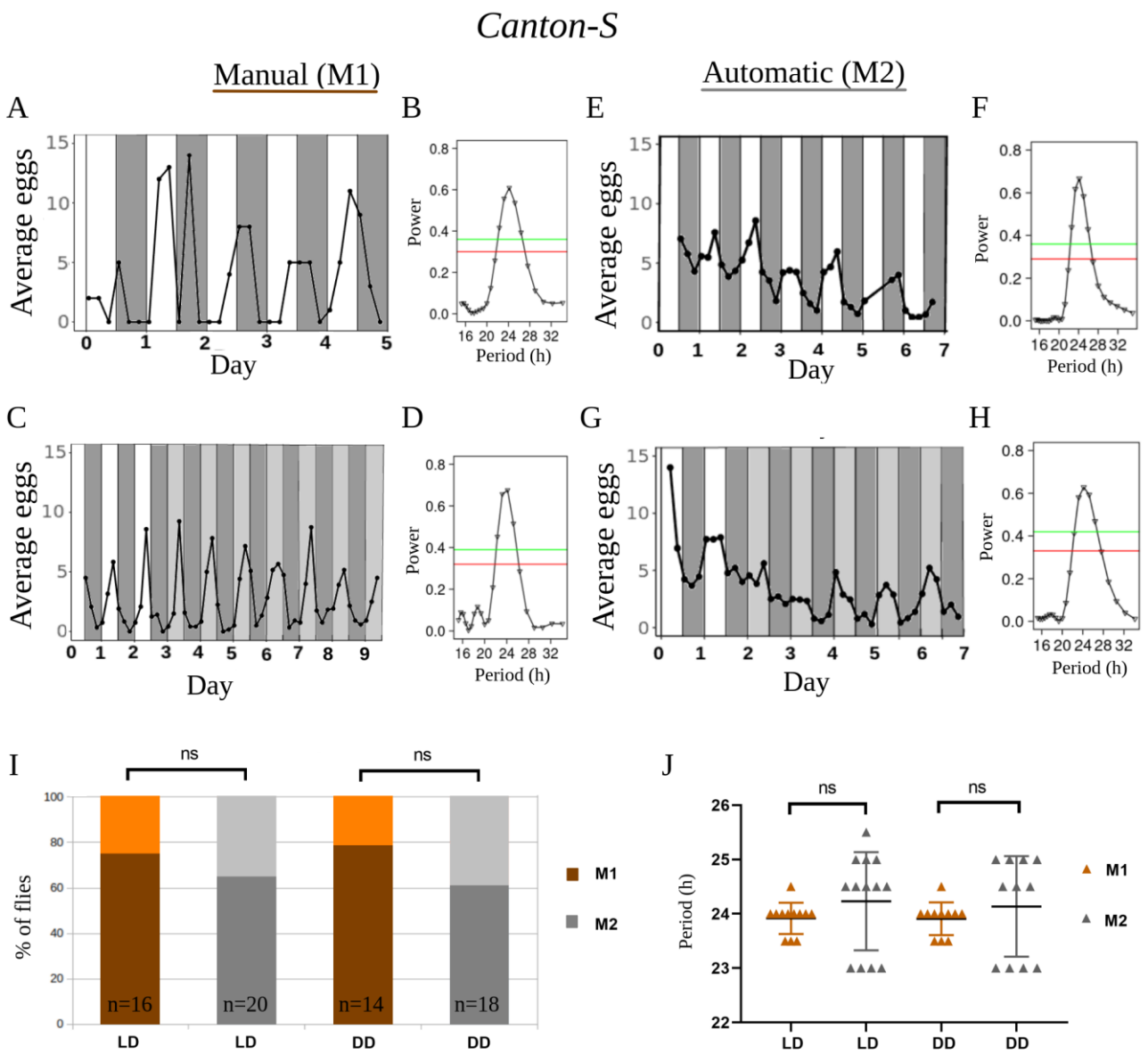

Figure 1. Manual and automatic egg collection methods give equivalent results. A, E: Average number of eggs laid by *Canton-S* females in 5 or 7 days of LD, obtained with the manual (A) or automatic (E) methods. **B, F**: Periodogram obtained for the egg time series depicted in panels A **(B)**, and E **(F)**. **C, G**: Average number of eggs laid by *Canton-S* females in 8 or 7 days of DD (with 2 or 3 days in LD, for training), obtained with the manual **(C)** or automatic methods. **D, H**: Periodogram obtained for the egg time series depicted in panels C **(D)**, and G **(H)**. **I**: Percent of rhythmic flies. **J**: Periods of rhythmic oviposition of individual flies. Mean ± SEM. indicated. Each dot represents one fly. ns: non significant (Unpaired t test).

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

Although it is widely accepted that egg laying in *Drosophila* is a rhythmic behavior, there is still no consensus about the mechanism that generates this rhythm. There was even an early report [18] about rhythmic egg laying in females lacking the protein PER (i.e. without a functional endogenous clock) which could not be confirmed by later studies [19]. Since these works used different methods to examine this behavior we decided to use our automatic approach to study egg laying in this mutant. As **Figure 2** shows, we found that oviposition in per^{01} females was completely arrhythmic in constant darkness. This supports the idea that egg laying is controlled by an endogenous circadian clock.

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 [20,21]. 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. Earlier work proposed that oviposition might also be controlled by a peripheral clock [13]. In order to test for this possibility, we used RNA interference (RNAi) to knock down the clock protein PER in all clock neurons in the brain (using the *Clk*856 driver [22]), thereby disrupting the molecular clock in these neurons. As shown in **Figure 2**, under these conditions 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 residual PER in clock cells, given the incomplete efficiency of RNA interference [23]. Given the wide range of periods observed in the few rhythmic flies found, we favor the second hypothesis.

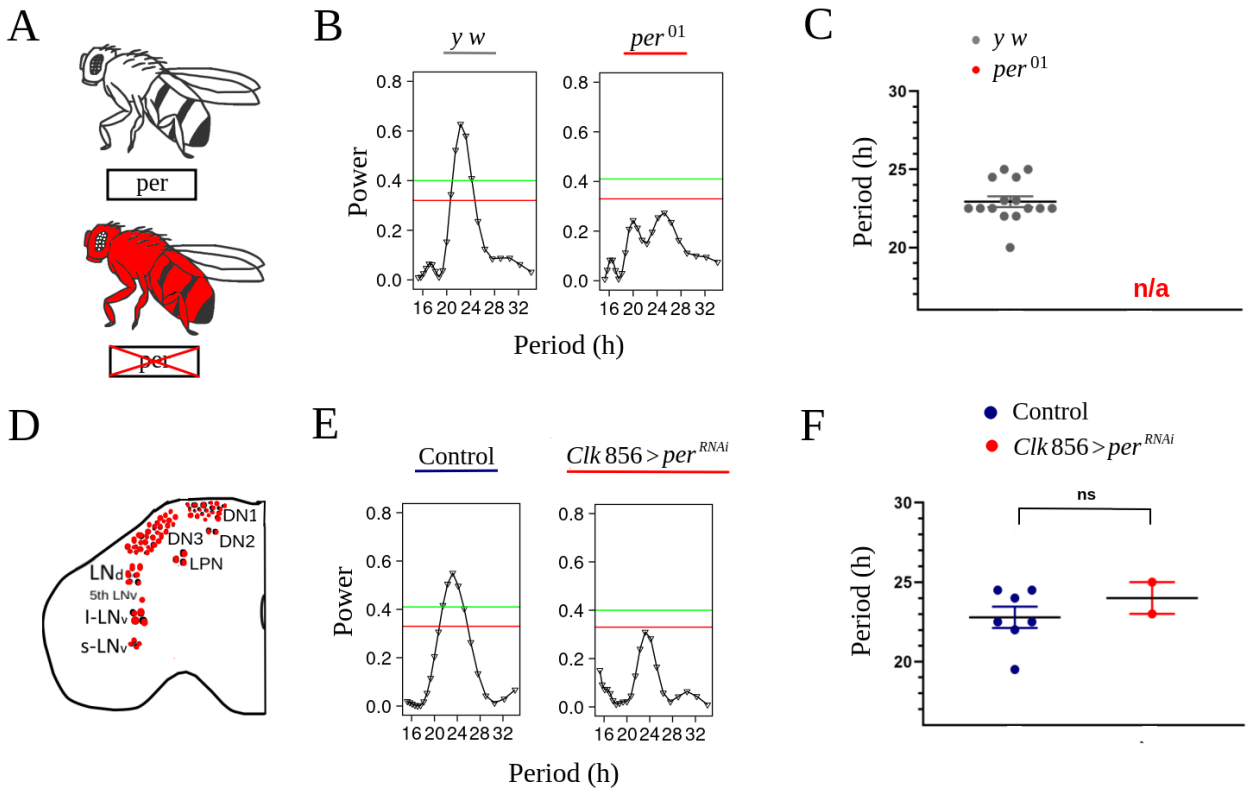

Figure 2. Disruption of the molecular clock in brain neurons eliminates circadian rhythmicity of oviposition. A, D: Schematic diagram of the regions (painted in red) where the molecular clock has been disrupted. **B, E**: Periodograms of the time series of the total number of eggs laid by females. Red and green horizontal lines represent significances of 0.05 and 0.01, respectively. **C, F**: Periods of rhythmic oviposition of individual flies. Mean ± SEM. are indicated. Each dot represents a single fly. B: *y w* (n=30), *per*^{01} (n=16). E: *Clk*856>*per*^{RNAi} (*Clk*856-Gal4>UAS-*per*^{RNAi}, n=26), Control (UAS-*per*^{RNAi}>+, n=22). ns: non significant (Mann Whitney test).

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 [8,24]. 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 3B**). This is in line with previous results describing rhythmic oviposition after ablation or electrically silencing all PDF+ neurons [14]. In addition, we observed a shortening of the egg-laying period when compared to controls (see **Figure 3F**), which suggests that these neurons may indeed 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 [25] and generation of evening activity [26,27] when the peak of egg deposition occurs. Interestingly, however, it has recently been shown that a posterior subset (DN1p) drive the circadian rhythm of oogenesis through the neuropeptide allatostatin C [28]. 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 [27]. As **Figure 3E** 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 [29]. Silencing DN1p neurons did not affect egg-laying in females which showed similar patterns (in terms of period and degree of rhythmicity) to those exhibited by controls females (see **Figures 3H and I**). Taken together, these results show that neither LNv nor DN1p neurons are essential for rhythmic oviposition.

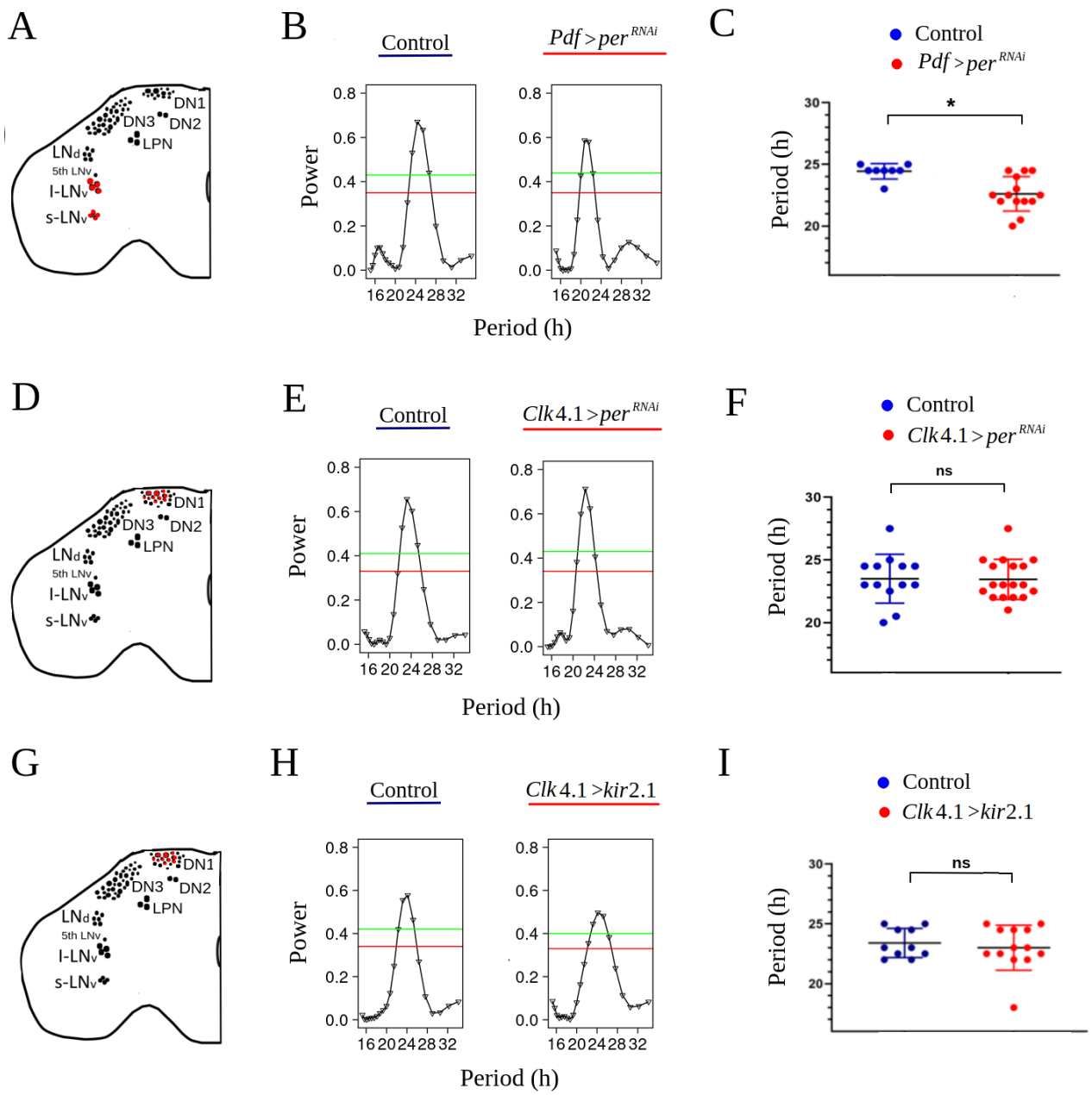

Figure 3. LNv and DN1 neurons are not necessary for egg laying rhythmicity. A, D, G: Schematic diagram of the clusters (painted in red) where the molecular clocks have been disrupted. **B, E, H**: Periodograms of the time series of the total number of eggs laid by females. Red and green horizontal lines represent significances of 0.05 and 0.01, respectively. **C, F, I**: Periods of rhythmic oviposition of individual flies. Mean ± SEM. indicated. Each dot represents one fly. B: *Pdf*>*per*^{RNAi} (*PdfDicer*-Gal4>UAS-*per*^{RNAi}, n=34), control (UAS-*per*^{RNAi}>+, n=18). **E**: *Clk*4.1>*per*^{RNAi} (*Clk*4.1-Gal4>UAS-*per*^{RNAi} , n=40), control (UAS-*per*^{RNAi}>+, n=38). H: *Clk*4.1>*kir*2.1 (*Clk*4.1-Gal4>UAS-*kir*2.1, n=35), control (UAS-*kir*2.1>+, n=36). ns: non significant, *: $p<0.05$ (Unpaired t test and Mann Whitney test).

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 [30,31]. 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 [32,33]. 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 [34]. 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 [35]. In order to assess the role of the Cry+ LNds in the control of oviposition, we resorted to the very specific MB122B driver [36,37] to perform an RNAi-mediated disruption of the molecular clock in those neurons. As **Figure 4B** 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 [6,38] 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 (see **Figure 4E**), 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 [39]. This would imply that locomotor activity and oviposition rhythms are controlled by distinct groups of clock neurons. However, Bulthuis et al. (2019) 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 [40] or the morning anticipation [41]. Thus, we examined 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 [42], 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 [41]). Interestingly, mated females with disrupted E cell clocks were as rhythmic as control flies (**Figure 5**), 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 prominent rhythmic behaviors are primarily controlled by distinct groups of clock neurons.

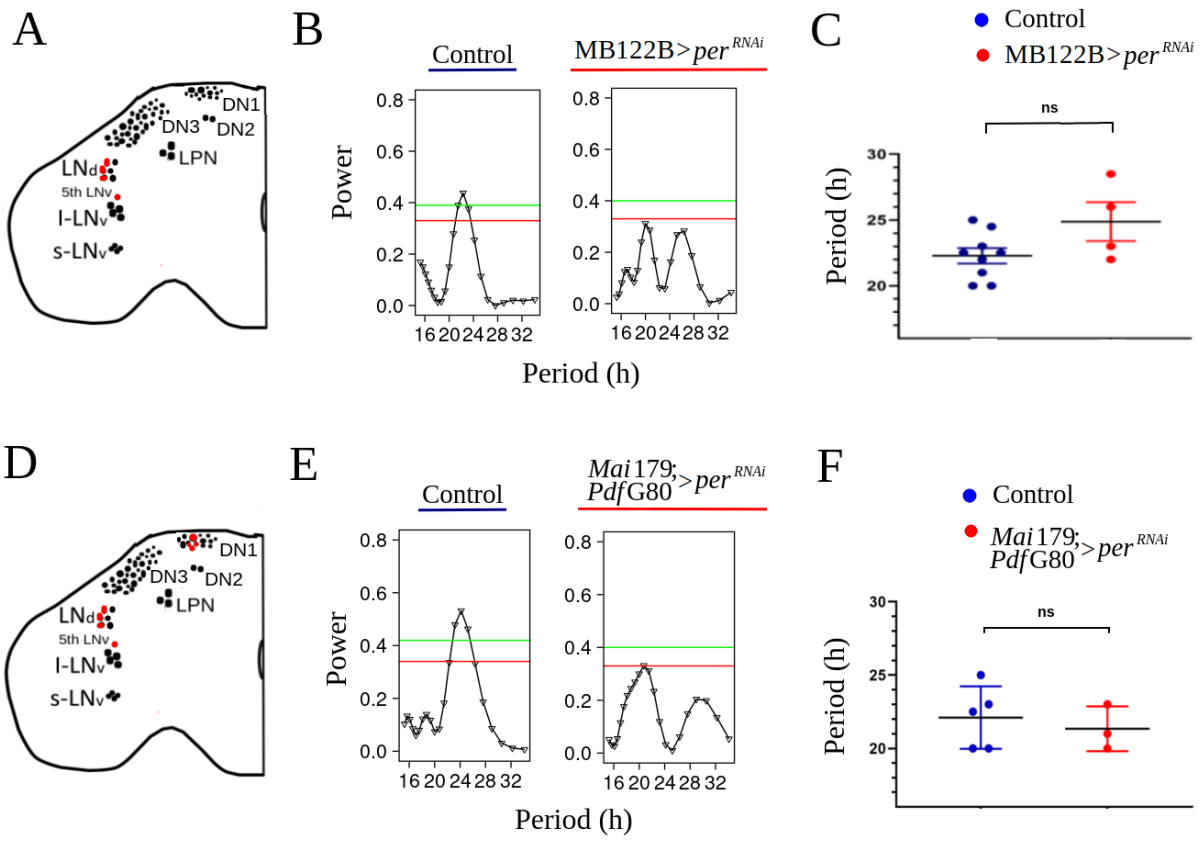

Figure 4. Disruption of the molecular clock in Cry+ LNd neurons drastically reduces the circadian rhythmicity of oviposition. A, D: Schematic diagram of the regions (painted in red) where the molecular clocks have been disrupted. **B, E**: Periodograms of the time series of the total number of eggs laid by females. Red and green horizontal lines represent significances of 0.05 and 0.01, respectively. **C, F**: Periods of rhythmic oviposition of individual flies. Mean ± SEM indicated. Each dot represents one fly. B: MB122B>*per*^{RNAi} (MB122B-splitGal4>UAS-*per*^{RNAi}, n=21), control (UAS-*per*^{RNAi} >+, n=25). E: *Mai*179;*Pdf*G80>*per*^{RNAi} (*Mai*179-Gal4;*pdf*-Gal80>UAS-*per*^{RNAi}, n=19), control (UAS-*per*^{RNAi}>+, n=14). ns: non significant (Mann Whitney test).

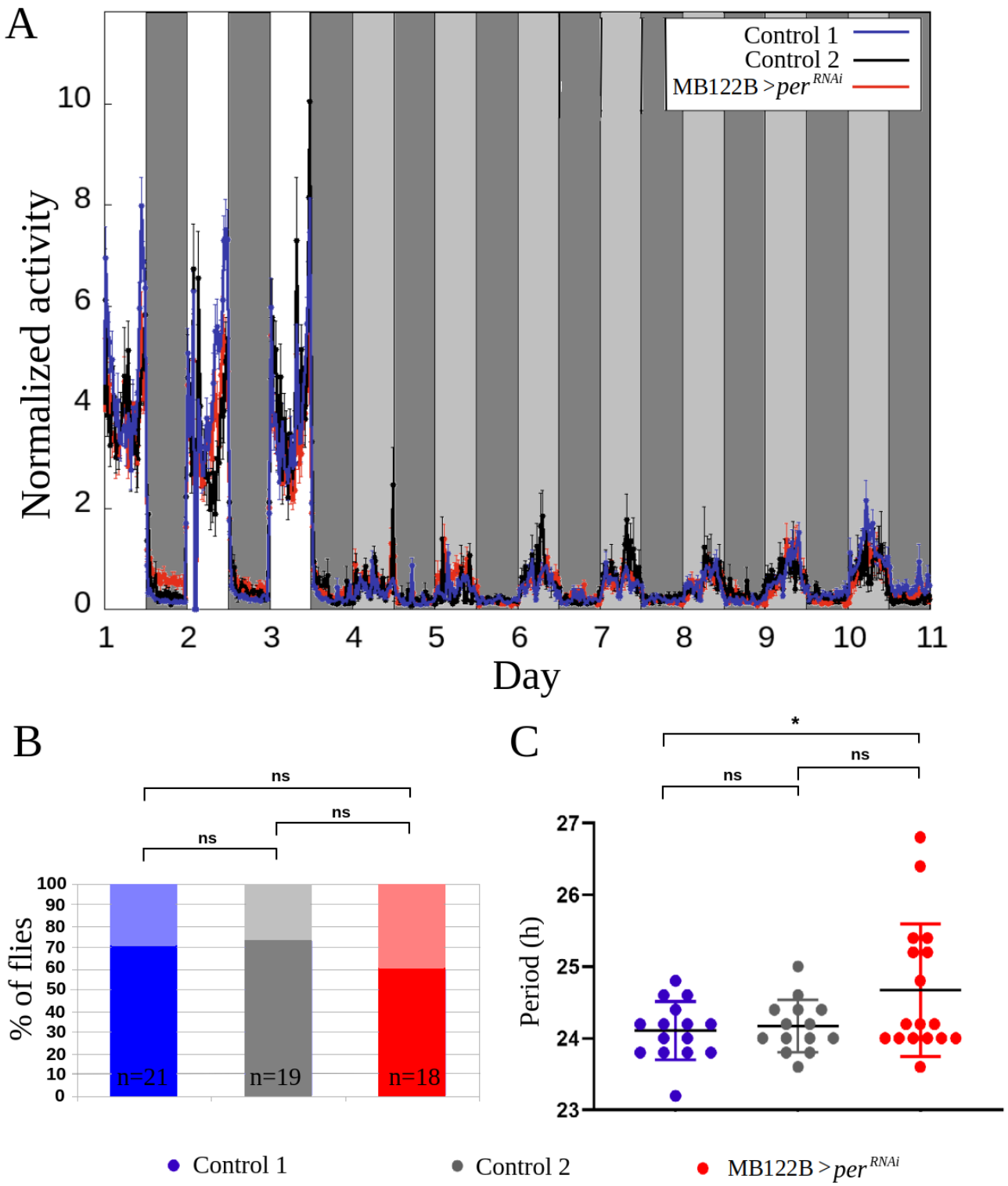

Figure 5. **Disruption of the molecular clock in Cry+ LNd neurons does not alter the circadian rhythmicity of locomotor activity. A**: Average activity recording of mated female flies during 3 days in LD and 7 in DD. Light grey bars represent subjective days. **B**: percent of rhythmic flies in DD. **C**: 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 [43–45], 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 [46] . 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[46]). 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 [45]. This makes a total of 25 oviposition-related neurons identified thus far.

To address the possibility of direct connections between circadian and neurons involved in egg laying behavior we probed the connectome for evidence of synaptic connections between clock and oviposition neurons and the full results are displayed in Tables S1 and S2. In short, we found that whereas there are no connections between LNv or DN1 neurons and oviposition neurons, LNds contact oviIN and pC1 neurons (see **Figure 6**), further supporting the experimental findings

described in previous sections. Interestingly, different subgroups of E cells [35] show distinctive patterns of connectivity: the E3 neurons (comprising the Cry- LNd1, LNd2 and LNd3) only contact the pC1 group, while the E2 neurons (comprising the Cry+ LNd4 and LNd5) only contact the oviIN neurons (see **Figure 5**). On the other hand, the E1 neurons (comprising the 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 the 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 [47] and the oviIN, with the strongest connections going from oviIN to LPN (**Supplementary Table 1 and Supplementary Figure 1**).

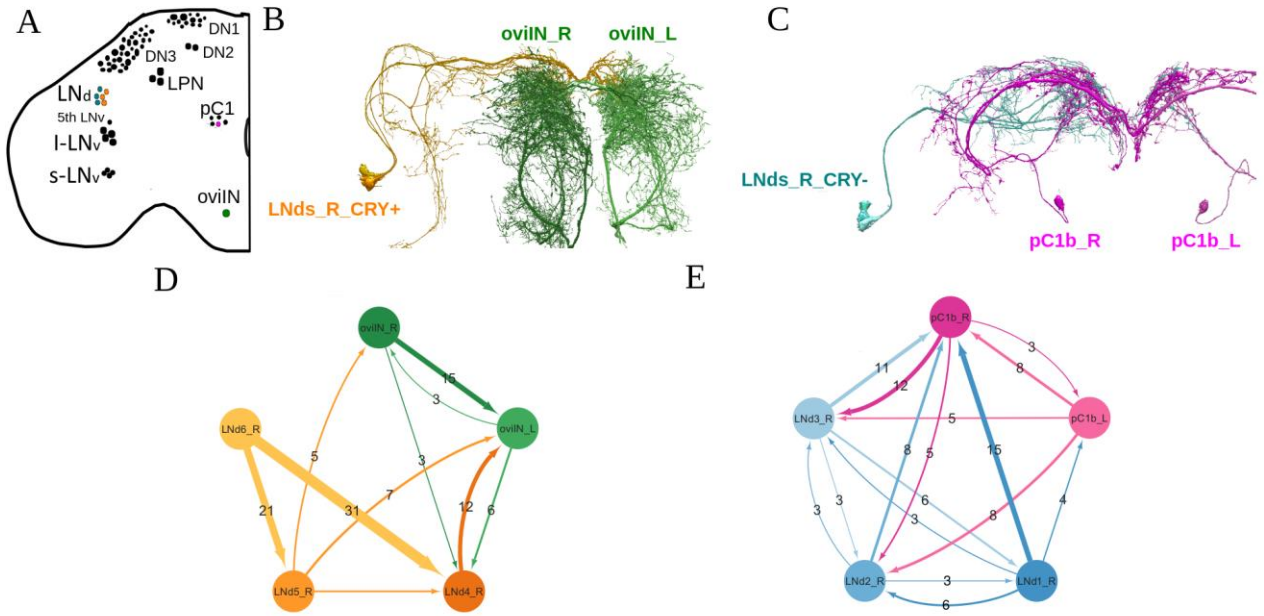

Figure 6. Direct synaptic connections between circadian clock neurons and oviposition-related neurons in the hemibrain dataset. A: Schematic diagrams showing the different clusters analyzed (in color). **B**: Connection between Cry+ LNd and oviIN neurons. **C**: Connection between Cry- LNd 2and pC1b 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

Egg-laying is one of the most important aspects of insect reproductive biology. Leaving an egg on a substrate represents the last decision a female insect makes in favor of her progeny. Consequently, the process of laying an egg, as well as the decision of when and where to lay an egg, carry a great deal of evolutionary weight [10]. This decision depends not only on external conditions, such as the suitability of the substrate as well as light and temperature cues, but also on the internal state of the fly, that in turn depends on the mating status, egg maturation, etc. It has been argued since the seventies that one of those internal signals is provided by the endogenous circadian clock.

The oviposition rhythm, however, 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 [48] .

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. Evidently, egg-laying is even less rhythmic in DD. This is to be expected, 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 data of a female 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 could be extracted by averaging the oviposition data. This led us to prefer periodograms of averages 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 [13,49], there is still no information about the relative importance of the different neuronal groups in driving this behavior. Using our automated method, 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 both *per* null mutants and 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. The apparent discrepancy with previous reports might rely on a rather permissive definition of rhythmicity (evidenced by the wide dispersion of the periods obtained; [18]).

We also disrupted the molecular clock (or electrically silenced) PDF-expressing neurons as well as in the DN1p group with no apparent effect on egg laying/oviposition rhythms, confirming previous reports [14]. 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 [6,34]. Interestingly, however, it has been recently shown that disrupting the molecular clock in these same neurons does not alter locomotor rhythmicity [50,51] suggesting a more complex scenario.

Somewhat surprisingly, the disruption of the molecular clock in Cry-expressing LNd neurons led to an almost complete loss of the oviposition rhythm. In contrast, (partially) ablating the clock in Cry+ LNds did not affect rhythmic locomotor activity in mated females either, 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, [36]), 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 [52,53].

The neural circuitry that controls the egg laying process has just begun to be unraveled [43–45]. 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 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 of the functional connections we uncovered (**Figure 7**). Furthermore, there is a clear separation of roles inside the LNd cluster, since the oviINs are contacted by only two Cry+ LNds (the so-called E2 cells [35]) and the pC1s are only contacted by the three Cry- LNds (E3 cells [35]). 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. 6**). The resulting clock/oviposition network is summarized in **Figure 7.**

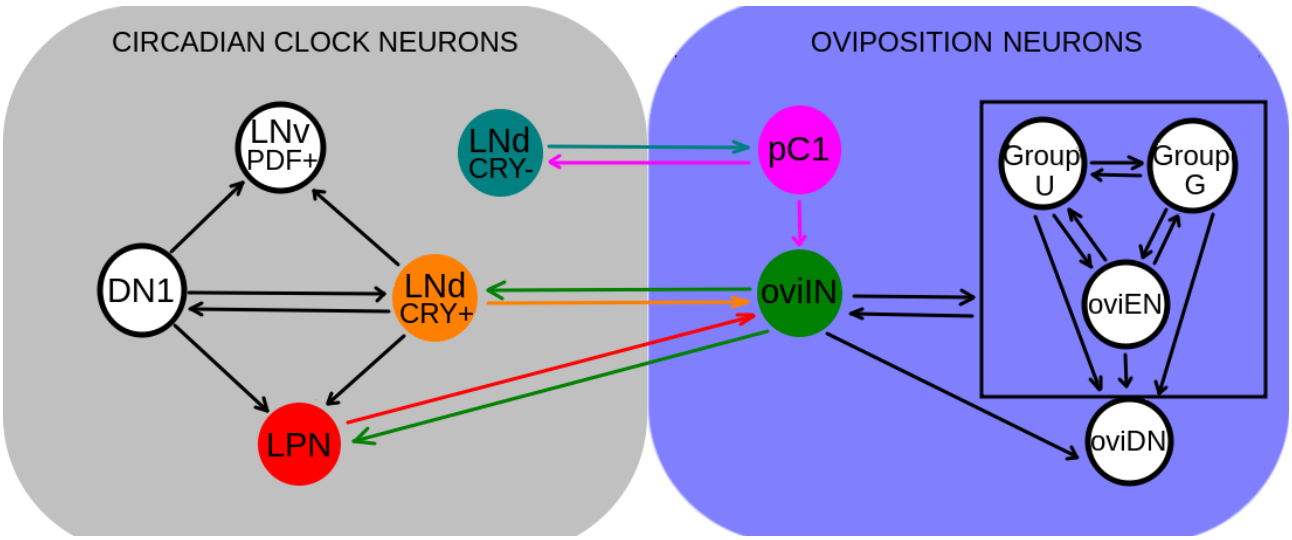

Figure 7. Scheme of connections between circadian clock and oviposition-related neuron clusters of the same hemisphere in the hemibrain dataset. Each circle represents a neuron cluster, comprising different numbers of neurons (some clusters comprise only one neuron). Clusters and connections involved 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 [45].

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 [44]. On the other hand, since pC1 neurons control female receptivity, it is tempting to speculate a time of day component to this behavior that could be accomplished through the E3-pC1b connection. In particular, the female mating rate has been shown to display circadian rhythmicity [54], and very recently it has been proposed that pC1b neurons are involved in the control of female copulation rate [55]. 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 [40,41,56].

The role of the LPN neurons in the circadian clock is still unclear [57] and therefore no clear role in rhythmic oviposition can be ascribed to the LPN-oviIN connection. On the other hand, since the connection is much stronger as input to the LPN (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].

***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 per01 w;	*Bloomington Drosophila Stock Center*	BDSC: 54	per^{01}
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-GAL4.P2.4}X, y[1]	*Bloomington Drosophila Stock Center*	BDSC:6899	*PdfDicer*-Gal4

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

Oviposition device

The device **(Figure S2)** 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 oviposition 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 of the track, 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 the 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 or 24 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 [41]. 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 form the video (with ad hoc programmed software) and registered. Locomotor activity of mated females was monitored for 3 days in LD conditions and then transfer to DD conditions for 7 days. Every 4 o 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. 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 [58,59]. 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 18 and 32 hs, and it was above the p=0.05 significance line.

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.) [41].

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 [46]. To access the data we used the NeuPrintExplorer Web tool (https://neuprint.janelia.org/). The result of our queries is summarized in **Tables S1 and S2**. 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. [35] . 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. [46]. 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 [46] we excluded them from the analyses. The figures that show connectivity between sets of neurons were drawn using Cytoscape version 3.10.1 [60].

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.