The Circadian Neuropeptide PDF has Sexually Dimorphic Effects on Activity Rhythms

Aishwarya R. Iyer[1], Eva Scholz-Carlson[1], Evardra Bell[1], Grace Biondi[1], Shlesha Richhariya[2], and Maria P. Fernandez[1,3]

1- Department of Neuroscience and Behavior, Barnard College. New York, NY 10027
2- Department of Biology, Brandeis University. Waltham, MA 02454
3- Corresponding author: mfernand@barnard.edu

Abstract

The circadian system regulates the timing of multiple molecular, physiological, metabolic, and behavioral phenomena. In *Drosophila* as in other species, most of the research on how the timekeeping system in the brain controls timing of behavioral outputs has been conducted in males, or sex was not included as a biological variable. The main circadian pacemaker neurons in *Drosophila* release the neuropeptide Pigment Dispersing Factor (PDF), which functions as a key synchronizing factor in the network with complex effects on other clock neurons. Lack of *Pdf* or its receptor, *PdfR,* results in most flies displaying arrhythmicity in activity-rest cycles under constant conditions. However, our results show that female circadian rhythms are less affected by mutations in both *Pdf* and *PdfR*. Crispr-Cas9 mutagenesis of *Pdf* specifically in the ventral lateral neurons (LN_vs) also has a greater effect on male rhythms. We tested the influence of the M-cells over the circadian network and show that speeding up the molecular clock specifically in the M-cells leads to sexually dimorphic phenotypes, with a more pronounced effect on male rhythmic behavior. Our results suggest that the female circadian system is more resilient to manipulations of the PDF pathway and that circadian timekeeping is more distributed across the clock neuron network in females.

Keywords: circadian rhythms, sex differences, PDF, neuropeptides, *Drosophila,* sleep/wake cycles

Introduction

The differences in neuronal circadian timekeeping between sexes remain relatively unexplored, despite the expanding body of research highlighting the influence of sex on the mechanisms underlying neuronal control of behavior [1]. In mammals, steroid hormones display daily, clock-driven changes in abundance, and while these sex hormones are not required to maintain rhythms, they influence the amplitude and response to photic stimuli [1, 2]. Furthermore, structural and functional sex differences have been observed in brain areas that receive direct input from the brain's circadian timekeeping center [2, 3]. Research in humans has also revealed significant sexual dimorphism: men tend to have lower-amplitude endogenous rhythms than women [4], and they are less resilient to nocturnal sleep disruptions and spend less time asleep [5].

In mammals, the main circadian pacemaker resides in the suprachiasmatic nuclei (SCN), which in mice consist of a network of ~20,000 neurons (reviewed by [6]). The *Drosophila* circadian clock neuron network involves ~150 neurons and is the functional equivalent of the mammalian SCN [7, 8]. Each circadian clock neuron contains an intracellular molecular timekeeping mechanism based on a transcriptional-translational feedback loop: the genes *Clock* (*Clk*) and *cycle* (*cyc*) promote rhythmic transcription of several key genes, including *Period* (*Per*) *and Timeless* (*Tim*), which inhibit their own transcription [9]. Multiple kinases that act on components of these clock proteins and can affect the pace of the molecular clock have been identified. One such kinase is *Doubletime* (DBT), which binds to and phosphorylates PER, hindering its nuclear accumulation [10, 11].

The fly's clock network consists of lateral neurons (LNs), which include ventroateral (LN_v), dorsolateral (LN_d), and lateral posterior neurons (LPNs), as well as three groups of dorsal neurons (DN1, DN2, and DN3), some of which can be further subdivided [8, 12-16]. The ventral and dorsal LNs are sufficient to produce the normal endogenous bimodal rhythm of sleep and activity [17, 18]. The four small LN_vs (s-LN_vs) are usually referred to as morning cells (M-cells) since they control the morning peak of activity under light-dark cycles (LD). These cells are also essential to maintain rhythmicity under free-running conditions [19, 20]. The evening peak is controlled by the LN_ds and a *Pdf*-negative LN_v, the 5th LN_v (E-cells) [17, 21, 22]. Some DNs also contribute to timing and amount of sleep via modulation of the M and E cells [23-27].

Release of the circadian neuropeptide Pigment Dispersing Factor (PDF) by the s-LN_vs is essential for endogenous circadian timekeeping. A *Pdf* null mutation, Pdf^{01}, results in a substantial fraction of arrhythmic flies [19], desynchronization of molecular oscillations [28, 29], and phase changes in electrical activity of some clock clusters, most notably the LNds [30]. Loss of *PdfR* also leads to loss of behavioral rhythms [31-33]. Interestingly, PDF and PDFR also regulate behaviors that are sex-specific or sexually dimorphic. Rival induced long mating duration requires PDF expression in the s-LN_vs, PDFR expression in a subset of LN_ds, and NPF expression in the LN_ds [34]. PDF controls rhythms in the sexually dimorphic pheromone profiles produced by the oenocytes [35], and is involved in long term mating suppression in males [36]. Both PDF and PDFR contribute to geotactic behaviors [37] and the phenotypes of Pdf^{01} mutants are sexually dimorphic, with males showing a more extreme negative geotaxis phenotype [38].

Sexual dimorphism in *Drosophila* sleep/wake cycles has been studied mostly under LD cycles. Males show lower activity and more sleep during the light phase [39-42]. This higher midday activity is due to the activity of a subset of sleep-promoting DN1s, which is more active in males [39] and receives input from the male-specific P1 neurons that control male courtship [40]. Unlike studies on circadian rhythms, *Drosophila* sleep research often involves only females. Males also have an earlier and more pronounced morning peak and a larger phase angle between the morning and the evening peak [43]. Under conditions of constant darkness and temperature (DD), males of several wild type strains have a small but significant reduction in free-running period relative to females of the same strain [43]. Moreover, males are more likely to retain a bimodal activity pattern in DD [43]. A recent transcriptomic analysis of *fruitless* (*fru*) expressing neurons revealed clusters that are enriched in circadian clock genes [44]. Interestingly, only the male dataset had some *fru*+ clusters with multiple genes having known circadian functions. A previous study reported that the DN1s express the male-specific Fru^M protein [45], and that the number of cells in the $DN1_p$s cluster is sexually dimorphic in cell number [46]. In addition, the E3 subset of LN_ds has been shown to be dimorphic in expression of the neuropeptide NPF [47, 48].

Given the sexually dimorphic roles of neuropeptides, including PDF, in other behaviors [49], we asked if females were similarly affected by manipulations of the *Pdf/PdfR* pathway. We found that

female circadian rhythms are less affected by null mutations in both *Pdf* and *PdfR*, and that similar effects are observed via CRISPR-Cas9 *Pdf* mutagenesis specifically in the LN_vs. Moreover, speeding up the molecular clock in the LN_vs via expression of DBT^s leads to an advance of the morning peak in males but not in females, and the pace of the free-running period of activity is significantly shortened only in males. Taken together, our results show that the female circadian system is more resilient to manipulations in the PDF pathway and suggest that the *Pdf*+ neurons play a more dominant role over the male than over the female circadian network.

Results

Mutations in PDF and PDFR lead to sexually dimorphic phenotypes.

A null mutation in *Pdf* results in molecular, physiological, and behavioral phenotypes in males [50]. We assayed the activity rhythms of Pdf^{01}females under free-running conditions (DD) and found that a remarkably large proportion of the experimental females were still rhythmic (Fig. 1 A-C). We calculated the difference in rhythmic power of the experimental flies relative to their respective controls and found that the effect is less pronounced in females than in males (Fig. 1 D-E), suggesting that experimental females have more consolidated rhythms. Mutant females that were still rhythmic had a slightly but significantly shorter free-running period than did the controls (Table 1). This phenotype, previously described in Pdf^{01} males, was not observed in experimental males, consistent with the findings of a recent study [51].

Pdf mutants have increased sleep, and this effect is mediated by PDF function on the LN_vs themselves [52]. We found that both sexes show an increase in total sleep in LD, but the effect is more pronounced in females (Fig. 1F-I). While the sleep increase in males was most prominent at midday, females exhibited increased sleep throughout most of the light phase (Fig. 1G). To rule out the presence of remnant PDF expression in Pdf^{01} females we stained brains of control and experimental males and females with an anti-PDF antibody. We did not observe any traces of PDF in experimental flies of either sex (Fig. S1A).

125 PDF accumulates rhythmically in the s-LN_v dorsal termini in a time-of-day dependent manner
126 both in LD and DD [19, 20], and can be released from the projections and the soma [53]. To
127 examine if there are differences in the phase and amplitude of PDF cycling between the sexes in a
128 wild type background, we dissected *Canton-S* males and females on the third day under DD at 6
129 timepoints over a 24-hour cycle. Using a COSINOR-based curve fitting method [54], we found
130 that both males and females have a significant 24-hour rhythms in PDF cycling in their dorsal
131 projections, with no significant sex differences in phase or amplitude (Fig. S1B-C, Table 2).
132
133 Next, we asked if the effects of a *Pdf* receptor mutation (*PdfR*) on activity and sleep were also
134 sexually dimorphic. Expression of PDFR, a GPCR, can be detected in most of the clock neurons
135 except for 3 LNds, half of DN1ps, and some DN3s [55], coinciding with *Cryptochrome* expression
136 in clock neurons [55]. However, *PdfR* mRNA appears to be expressed in all clock neurons [56].
137 The han^{5304} mutant is a *PdfR* hypomorph and exhibits behavioral phenotypes under both LD 12:12
138 and DD [31-33]. Under DD, both *Han* males and females had a significant reduction in rhythmicity
139 compared to the controls, but there was a greater proportion of rhythmic females (~65%) than
140 males (~16%) (Fig. 1J). The free-running period of experimental flies was significantly shorter for
141 both sexes (Table 3), as reported previously for males. Rhythmic power is significantly lower than
142 controls for both han^{5304} males and females (Fig. 1K), but the effect was more pronounced in
143 males, suggesting that females have more consolidated rhythms (Fig. 1L). Sleep plots for the first
144 week under DD show that both males and females sleep more than controls (Fig. 1E). Similar to
145 the effect of the *Pdf* mutation, han^{5304} flies showed significantly higher levels of LD sleep
146 compared to controls, and the effect was again more pronounced in females (Fig. 1 M-O). Taken
147 together, the results suggest that female circadian rhythms are less affected by the loss of both *Pdf*
148 and *PdfR*, while at least under LD, the sleep phenotypes are more pronounced in females.
149
150 **CRISPR-Cas9 mediated *Pdf* mutagenesis has more pronounced effects on male behavior.**
151
152 In the *Pdf* null mutant background, other genetic factors could contribute to the sexual
153 dimorphism observed in behavioral rhythms. We therefore employed a tissue-specific CRISPR-
154 Cas9 mediated knockout of *Pdf* in both males and females, as described in a recent study that
155 focused on males [57]. To assess the efficiency of the manipulation we stained for PDF in flies that

156 constitutively expressed *Pdf* gRNA and Cas9 in *Pdf*+ neurons. This experiment was conducted at
157 28°C, as this temperature was more effective at mimicking the behavioral phenotypes of the Pdf^{01}
158 mutant than was 25°C (described below), and to allow direct comparison with an adult-specific
159 manipulations.
160
161 PDF was significantly reduced in the s-LN_vs in both sexes (Fig. 2 A-B). However, in most brains
162 we noted faint staining in the dorsal projections of at least one s-LN_v in at least one hemisphere.
163 The PDF signal within the large LN_vs was higher and could be detected in two or three l-LNv cell
164 bodies in both hemispheres in the majority of brains. In addition to behavioral phenotypes, the
165 Pdf^{01} mutation leads to pronounced misrouting of the s-LN_v projections in male flies [58]. We next
166 employed a transgene expressing a red fluorescent protein under a *Pdf* regulatory sequence [59]
167 and observed some s-LN_v projections occasionally defasciculating from the main axonal bundle
168 in one or both hemispheres in *Pdf >Pdf*-g; *Cas9* flies of both sexes (Fig. 2A). The extent of the
169 reduction in PDF intensity of experimental flies relative to the controls was similar between the
170 sexes (Fig. 2C).
171
172 We then analyzed activity-rest rhythms in *Pdf >Pdf*-g; *Cas9* flies and found that experimental
173 males were mostly arrhythmic, whereas a greater proportion of experimental females remained
174 rhythmic (Fig. 3A-B). The free-running period of experimental males was not significantly
175 different from one of the controls, possibly because of the wide range of periods exhibited. Females
176 had significantly shorter free-running period compared to parental controls (Fig. 3C). The
177 rhythmic power was significantly lower in experimental flies of both sexes (Fig. 3D) but the effect
178 was less pronounced in females (Fig. 3E), indicating that females are able to better consolidate
179 activity rhythms. At 25°C, both males and females exhibited reduction in percent rhythmicity but
180 the majority of the flies were rhythmic (Fig S3A). Both males and females showed a shortening of
181 their free-running period (Fig. S3B). Similar to what we observed at 28°C, the rhythmic power of
182 the experimental flies of both the sexes was significantly reduced (Fig. S3C) but females were less
183 affected (Fig. S3D).
184
185 We next conducted an adult specific Crispr-Cas9 knockdown of *Pdf* (Fig S4). While a previous
186 study conducted in males did not find a correlation between the misrouting induced by the loss of

187 *Pdf* during development and the loss of behavioral rhythms in adults [60], it is possible that the
188 sex differences in $Pdf^{01}$and *Pdf* >*Pdf*-g; *Cas9* flies were due to developmental effects. The AGES
189 system was previously employed to rule of a developmental contribution to the *Pdf* mutagenesis
190 phenotypes [57], but a recent study showed that AUXIN exposure short- and long-term changes
191 in physiology and behavior [61]. Therefore, we employed a temperature-sensitive Gal80 variant
192 with ubiquitous expression to conditionally inhibit Gal4-mediated expression of the *Pdf* guide and
193 Cas9 [62]. This allows for the temporal control of UAS transgenes since $Gal80^{ts}$ is active at lower
194 temperatures but inactive at higher temperatures. We raised flies at 18˚C and transferred them to
195 28˚C immediately after eclosion, so that *Pdf* mutagenesis only occurs in adult flies. Consistent
196 with what was reported for males by Gorostiza *et al*. [60] using RNA mediated interference (RNAi)
197 of *Pdf*, we did not observe misrouting in flies of either sex (Fig. S4A). While PDF levels were
198 significantly reduced in both males and females, the manipulation was more effective in males
199 (Fig S4A-B). Therefore, although the circadian behavioral phenotypes were again more
200 pronounced in males than in females (Fig S4C-E), this is likely due to higher remaining PDF
201 expression in experimental females.
202
203 **Speeding up the M-cell clock leads to a coherent period shortening only in males**
204
205 Next, we sought to determine if the influence of the *Pdf* releasing cells themselves was sexually
206 dimorphic. While PDF is released from both the large and small LN_vs, only the s-LN_vs (M-cells)
207 play key roles in regulating free-running rhythm properties [17]. In males, manipulations that
208 change the pace of the clock specifically in the LN_vs result in changes in the phase of the morning
209 peak of activity and in free running period [21, 63]. We expressed the *Doubletime* 'short' (DBT^s)
210 allele [64] under the *Pdf-Gal4* driver (Fig. 4A) and analyzed the effects on behavior in both sexes.
211 Interestingly, we found that *Pdf* > DBT^s males but not females have an advanced phase of the
212 morning peak of activity (M-peak, Fig. 4B-C). This suggests that under LD cycles, the M-oscillator
213 is more effective at setting the phase of male than female behavior.
214
215 Under free-running conditions, both *Pdf* > DBT^s males and females flies showed a significantly
216 lower percentage of rhythmic flies compared to their controls (Fig. 4D-E), but there were fewer
217 rhythmic females (~40%) than males (~65%) (Fig. 4D). A possible reason for this could be that

218 the rest of the clock network, where the pace of the molecular clock remained unaltered, is able to
219 resist the influence of the M-cells to a greater extent in females. The free-running period (FRP) of
220 *Pdf > DBT*^{s} males was ~18.5 hours, with very few exceptions (Fig. 4F). However, *Pdf > DBT*^{s}
221 females showed a bimodal distribution, with some individuals showing a FRP consistent with the
222 pace of the M-cells (~18-18.5 hrs.), whereas a large proportion of females had FRP of ~24 hours,
223 consistent with the pace of the molecular clock in the rest of the clock network, which was not
224 affected by the genetic manipulation (Fig. 4F). Among rhythmic flies, both *Pdf > DBT*^{s} males and
225 females had lower rhythmic power than the controls (Fig. 4G), with no difference between the
226 sexes (Fig. 4H). These results support the notion that M-cells are more dominant over the male
227 than the female circadian network. Previous studies have shown that M-cells' influence over the
228 rest of the clock neurons is mediated via PDF [65], so it is possible that the differential role of M
229 cells in males and females is precisely due to the sexually dimorphic effects of PDF.
230
231 **Speeding up the E-cell clock leads to a more advanced evening peak in females**
232
233 We next asked if changing the pace of the clock in the evening cells (E-cells) via DBT^{S}
234 expression also had sexually dimorphic effects on behavior. These cells can be subdivided into at
235 least 3 different clusters, based on their anatomy [8], physiology [63], transcriptomics profiles [14]
236 and connectivity patterns [16]. The PDFR-expressing E1 and E2 clusters have been shown to
237 regulate evening activity under LD [65, 66] and to be able to maintain free-running activity
238 rhythms in the absence of a functional clock in the M cells [67, 68], while the behavioral role of
239 the E3 cluster remains unknown. We used the MB122-B split-gal4 driver to target the E1 and E2
240 subsets (Fig. 5A). We found that expressing DBT^{S} in the E1+E2 subsets of evening cells
241 significantly advanced the phase of E-peak in experimental flies of both sexes (Fig. 5B-C).
242 Remarkably, this effect was more pronounced in females (Fig. 5D).
243
244 Speeding up of clocks in the E1 + E2 LN$_{d}$s did not significantly alter the free-running period or
245 rhythmic power of experimental flies of either sex (Fig. 5E-F). M-cells have been shown to be the
246 most dominant oscillators in DD and to regulate rhythm properties like persistence and free-
247 running period to a large extent [17, 21, 22, 63], although manipulations of other clock cells can
248 affect rhythm properties to some extent. Thus, speeding up the clock in the PDFR^{+} E1 and E2

clusters leads to similar behavioral phenotypes in males and females under free-running conditions.

Discussion

The critical role of sex as a biological variable has gained increasing recognition in biomedical research [69, 70]. Male bias is particularly prevalent in neuroscience, with single-sex studies using male animals outnumber those using females by a ratio of 5.5 to 1 [71]. This disparity extends to chronobiology, leading to a lack of knowledge regarding how temporal organization in the nervous system is influenced by sex. However, work from several laboratories has revealed sexual dimorphism in the SCN, as well as in its input and output pathways [1, 72]. Sex differences in the SCN morphology have been described in both animal models and humans, as well as sex differences in SCN electrical activity and steroid hormone receptors. In addition, sex differences in the number of SCN neurons that express the key neuropeptide vasoactive intestinal polypeptide (VIP) and in *Vip* mRNA *e*xpression have also been reported (reviewed in [2]).The roles of the mammalian VIP and the *Drosophila* PDF to circadian physiology are highly similar, although neither these peptides nor their receptors are sequence orthologs [73].

Several studies over the years have shown the importance of PDF in generating coherent rhythms of ~2hour periodicity. Here, we report that females lacking *Pdf* or its receptor *PdfR* are more likely to be able to maintain consolidated activity-rest behavior than males. This could be because of sex differences in PDF signaling mechanisms, PDFR expression, or the roles of other clock neurons within the network. We did not detect sex differences in the overall levels or temporal pattern of PDF accumulation, suggesting that these differences might not contribute to the sexually dimorphic effects of the loss of *Pdf* expression. Another possibility is that in the female circadian system, other neuropeptides or neurotransmitters can maintain network synchronization even in the absence of PDF. In males, other neuropeptides have been shown to act in concert with PDF to maintain consolidated rhythms in the network, although none of them have as profound an effect as PDF in regulating activity-rest rhythms in DD [51, 74]. Single mutants of DH31 and CCHamide1 do not affect activity rhythms by themselves but the double mutants of these neuropeptides along with Pdf^{01} ($DH31^{01}Pdf^{01}$ and $Pdf^{01}/CCHa^{SK8}$) are almost completely arrhythmic, indicating that these neuropeptides act hierarchically in the network, with PDF being on top of that hierarchy [51, 74]. The importance of PDF relative to other peptides released by clock neurons may also be sexually dimorphic.

To overcome possible pleiotropic effects of *Pdf*^{01} and *PdfR* mutations, we employed a constitutive knockout of *Pdf* in the ventral lateral neurons using the CRISPR-Cas9 method. Although the Crispr manipulation was only partially effective at eliminating PDF expression, it was able to replicate the phenotypes of the *Pdf*^{01} mutation. We observed faint staining in the dorsal projections of at least one s-LN$_v$ in at least one hemisphere in most brains, and PDF staining in a single s-LN$_v$ projection reaching the dorsal brain has been shown to be sufficient for behavioral rhythms [75]. Experimental flies in which *Pdf* was mutagenized starting at the onset of the promoter expression early in development showed extensive misrouting of their dorsal termini, similar to what has been reported for *Pdf*^{01} males [60]. Instances of s-LN$_v$ misrouting have also been observed in other core clock mutants such as *per*^{01} and *tim*^{01} [76], and *cyc*^{01} [77]. We did not find evidence of sex differences in the severity of the misrouting phenotype caused by the loss of *Pdf,* but the percentage of brains with misrouting was higher in experimental females (data not shown), and no correlation between misrouting and behavioral phenotypes was found by others for *pdf*^{01} males [60]. Importantly, the *Pdf > Pdfg;Cas9* manipulation recapitulates the sexually dimorphic circadian phenotypes of *Pdf*^{01} mutants: a larger fraction of females are rhythmic and females exhibit a higher rhythm power.

In males, changing the speed of the M cell clock leads to phase changes in the morning peak under LD [65]. To determine if M-cell manipulations also have sexually dimorphic effects on behavior, we sped up the molecular clock by expressing the *Doubletime short* allele, *DBT*^{s}. Surprisingly, our results showed that speeding up the clock of M-cells advances the phase of the morning peak in males, while the female morning peak phase is not affected. These results support previous studies conducted in males on the role of M-cells in regulating the morning peak of activity [17, 22, 65], and suggest that the M cells are unable to regulate the phase of the morning activity in the same way in females. In DD, males had largely coherent short period rhythms and the majority (65%) were rhythmic. In contrast, only 40% of the females were rhythmic, and their FRP showed a bimodal distribution, with most flies exhibiting a ~24-hour period. This further supports the notion that the M-cells are less dominant over the circadian clock network in females. One possible explanation is that other clock neurons are able to “resist” their influence, and the

conflict between the fast-paced M cell clock and the ~24 clock other clock neurons is what leads to higher arrhythmicity in the females.

The Cryptochrome and *PdfR*-expressing clusters of evening cells – the sNPF expressing E1 cluster and the ITP-expressing E2 cluster [78] – have roles in setting the phase of the E-peak under LD and sustaining behavioral rhythms in the absence of a functional molecular clock in the M-cells [67, 68]. To test if these cells have a differential influence over the network in males and females, we expressed DBT^S under a driver that is expressed specifically in the E1 and E2 subsets of LN_ds. Our results showed that speeding up the clocks in the E1+E2 clusters resulted in a phase advance in evening peak of activity in both the sexes, but the effect was more pronounced in the females. Neither MB122 > DBT^S males nor females had phenotypes under free running conditions. A possible reason for the behavioral differences seen between the sexes could be redundancy in females, such that the network is not as dependent on PDF and therefore on M-cells for timekeeping. This would mean that the female network could have more distributed mode of timekeeping throughout the circadian clock network.

Across species, sex differences in the circadian timing system are largely related to the regulation of reproduction relevant behaviors. In mammals, the SCN determines the timing of the release of reproductive hormones and influences timing of mating (reviewed in [72]) and aggression [79] . In *Drosophila*, the circadian clock controls the timing of sex-specific and sexually dimorphic behaviors, such as male courtship [80] and aggression [81] and female sexual receptivity [82] and egg-laying [83]. This regulation of rhythmic behaviors requires connectivity between clock neurons and downstream sex specific circuits. For example, the $DN1_p$ cluster, which has been shown to be more active in males [39], is functionally connected to the male-specific *fru*-expressing P1 neurons that regulate male courtship [40]. In females, Allostatin C- producing $DN1_p$s have been shown to connect to downstream targets to control rhythms in oogenesis [84], and the Janelia female hemibrain connectome revealed that the LN_ds form connections *doublesex*-expressing PC1 cluster [16]. Our data suggest that the relative hierarchy of circadian oscillators is sexually dimorphic, with a less dominant M oscillator in females. This pattern of circadian timekeeping may serve an adaptive purpose, ensuring precise timing of essential female-specific behaviors crucial for reproductive fitness, such as sexual receptivity and egg laying.

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Materials and Methods

Fly lines and rearing

All genotypes were reared on standard cornmeal medium under LD (12 hr Light: 12 hr Dark) cycles and 25°C, unless specified otherwise (see figure legends for details). The fly lines used in this study were *Canton-S*, w^{1118}, Pdf^{01}, $PdfR^{01}$, *w*; *Pdf-RFP, Pdf-Gal4; tub-Gal80*^{ts}, *w*;UAS-*Cas9;* UAS-*Pdfg*, w; *Pdf-Gal4, w;;*UAS-*DBT*^{s}*,* and *w;MB122B-Gal4;*.

Activity-rest behavior recording and analysis

Individual male and virgin female flies (3–5 days old) were housed in glass locomotor tubes with sucrose agar food on one end and yarn on the other end. Locomotor activity was recorded using the Drosophila Activity Monitors (DAM, Trikinetics, Waltham, United States of America). Experiments were conducted in Tritech or Percival incubators under controlled light and temperature conditions. Flies were entrained to 12:12 LD cycles for at least 5 days, and then transferred to constant darkness (DD) for at least 7 days, at a constant temperature of 25°C, unless otherwise specified (see figure legends for details). Raw data obtained from the DAM system were scanned and binned into activity counts of 15 min interval using DAM File scan. Data was analyzed using the CLOCKLAB software (Actimetrics, Wilmette, IL).

Values of period and rhythmic power were calculated for a period of 8 days using the Chi-square periodogram with a cut-off of $p = 0.01$. The Rhythmic Power for each designated rhythmic fly was determined by subtracting the Chi-squared significance value from the Power of the Periodogram. Flies that did not exhibit a periodicity peak above the significance threshold were categorized as "arrhythmic," and their period and rhythmic power were not included in the analysis. Values of the Morning and Evening peaks was calculated using the PHASE software [85]. Total LD sleep values for all genotypes were calculated for a period of 3 days (LD days 2-4). Representative actograms were generated using ClockLab and activity plots were generated using PHASE. The period, rhythmic power, total sleep, phase values of all the flies for a particular experimental genotype were compared against the background or parental controls either using the Mann-Whitney test or the Kruskal-Wallis ANOVA followed by the Dunn's multiple comparisons test. The details on the statistical comparisons and the no. of flies used in a given experiment are indicated in their

respective figure legends. The number of rhythmic flies of the experimental genotype were compared against their respective background or parental controls using the Fisher's exact test. All statistical analysis was done using GraphPad Prism 9.0.

Immunohistochemistry

Brains from adult male or female flies (6-8 days old) were dissected in ice-cold Schneider's Insect media (S2) and fixed immediately after dissection in 2%Paraformaldehyde (PFA) in S2 media for 30 minutes at room temperature. The fixed brains were washed (3 washes of 10 mins each) with 0.3% PBS-Triton X 100 (PBS-TX) and then treated with the blocking solution (5% Normal Goat Serum made in 0.3% PBS-TX) for 1-hr at room temperature. The brains were then incubated with primary antibodies at 4°C for 24-48 hrs. The primary antibodies used were anti-PDF (mouse, 1:3000, C7, DSHB), anti-RFP (rabbit, 1:2000, Rockland). After incubation, the brains were given 6 washes with 0.3% PBS-TX and incubated with Alexa-fluor conjugated secondary antibodies overnight or for 24-48 hrs. at 4°C. The following secondary antibodies were used, goat anti-mouse 488 (1:3000, Invitrogen), goat anti-rabbit 568 (1:3000, Invitrogen). After incubation, the brain samples were washed 6 times with 0.3% PBS-TX, cleaned and mounted on a clean, glass slide using Vectashield mounting media.

Image acquisition and analysis

The slides were imaged using a confocal microscope (Olympus FV3000) using the 20X objective. Image analysis was performed using the Fiji software [86]. In the samples, small and large ventral lateral neurons were classified based on their anatomical locations and expression of PDF. PDF intensity in these cells were measured by selecting the slice of the Z-stack that showed the maximum intensity, drawing a Region of Interest (ROI) around the cells and measuring their intensities. 3–4 separate background values were also measured around each cell and the final intensity was taken as the difference between the cell intensity and the background. For quantification of PDF in the dorsal projections, a rectangular box was drawn as ROI starting from the point where the PDF projection turns into the dorsal brain and intensity was measured. 3–4 background values were also measured around the projection. The intensity values obtained from both the hemispheres for each cell type for each brain was averaged and used for statistical analysis. PDF intensity from the s-LN_v were compared between the experimental and control

genotypes using the Mann-Whitney test. To estimate different aspects of rhythmicity in PDF oscillations in the dorsal termini of s-LN_v in males and females, we used a COSINOR based curve-fitting method [54] COSINOR analysis was implemented using the CATCosinor function from the CATkit package written for R [87].

Figure legends

Figure 1: Mutations in *Pdf* and *PdfR* lead to sexually dimorphic phenotypes. (A) Representative actograms of w^{1118} (left) and Pdf^{01} (right) male flies for 6 days of LD followed by 7 days of DD. **(B)** Representative actograms of w^{1118} (left) and Pdf^{01} (right) female flies for 6 days of LD followed by 7 days of DD. **(C)** Percentage of rhythmic flies are plotted for control (w^{1118}) and Pdf^{01} males (left, n = 86 (w^{1118}), n = 81 (Pdf^{01})) and females (right, n = 75 (w^{1118}), n = 101 (Pdf^{01})). Error bars are SEM values plotted across 3 replicate experiments. **(D)** Rhythmic power of control (w^{1118}) and Pdf^{01} male and females calculated using the Chi-square periodogram are plotted. **(E)** The difference in rhythmic power of experimental males and females from their respective controls are plotted. **(F)** Average sleep plots of flies over 7 days in DD are plotted for male and female control (w^{1118}) and experimental (Pdf^{01}) flies. **(G)** Average sleep plots under LD 12:12 of control (w^{1118}) and experimental (Pdf^{01}) are plotted for males (left) and females (right). The plots are averaged over flies and days for a period of 3 days under LD 12:12. **(H)** Total sleep values under LD are plotted for male (left) and female (right) flies of control (w^{1118}) and experimental (Pdf^{01}) flies. **(I)** The difference in total LD sleep values of experimental males and females from their respective controls are plotted. **(J)** Percentage of rhythmic flies are plotted for control (w^{1118}) and han^{5304} males (left, n = 86 (w^{1118}), n = 115 (han^{5304})) and females (right, n = 74 (w^{1118}), n = 94 (han^{5304}))**.** **(K)** Rhythmic power of control (w^{1118}) and han^{5304} male and females calculated using the Chi-square periodogram are plotted. **(L)** The difference in rhythmic power of experimental males and females from their respective controls are plotted. **(M)** Average sleep plots under LD 12:12 of control (w^{1118}) and experimental (han^{5304}) flies are plotted for males (left) and females (right). The plots are averaged over flies and days for a period of 3 days under LD 12:12. **(N)** Total sleep values under LD are plotted for male (left) and female (right) flies of control (w^{1118}) and experimental (han^{5304}) flies. **(O)** The difference in total LD sleep values of experimental males

453 and females from their respective controls are plotted. Free-running period and rhythmic power
454 values are calculated using Chi square periodogram in CLOCKLAB for a period of 7 days. Total
455 sleep values are calculated for 3 days in LD using PHASE. Statistical comparisons were performed
456 between the control and experimental flies for both sexes using the Mann-Whitney U test. The box
457 plots extend from the 25th to 75th percentile, with whiskers extending from the smallest to the
458 largest value, and each point representing data from a single fly. Combined data from at least 3
459 replicate experiments are plotted.
460
461 **Figure 2: Tissue-specific CRISPR mediated *Pdf* manipulation leads to a reduction in PDF**
462 **levels and misrouting of s-LN$_v$ dorsal termini. (A)** Representative confocal images of control
463 (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts}*) (top) and experimental (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts} >*
464 *Cas9; Pdf-g*) (middle) male flies stained with RFP and PDF antibodies. Representative confocal
465 images of experimental (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts} > Cas9; Pdf-g*) (bottom) female flies
466 stained with RFP and PDF antibodies. The experimental flies show significant reduction in PDF
467 levels and misrouting of the s-LN$_v$ dorsal termini (arrows) as seen in the RFP channel for both the
468 sexes. **(B)** Quantification of PDF intensity from s-LN$_v$ cell bodies in control (*Pdf-RFP, Pdf-Gal4;*
469 *tub-Gal80^{ts}*) and experimental (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts} > Cas9; Pdf-g*) flies are plotted
470 for males (left) and females (right). Data points represent intensity from a single brain sample. $n >$
471 12 brains for all genotypes. **(C)** The difference in PDF intensity values of experimental males and
472 females from their respective controls are plotted.
473
474 **Figure 3: CRISPR-Cas9 mediated *Pdf* mutagenesis has more pronounced effects on male**
475 **behavior. (A)** Representative actograms of control (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts}*), and *(UAS*
476 *Cas9; Pdf-g)* and experimental (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts} > Cas9; Pdf-g*) males (left) and
477 females (right) are plotted for 6 days of LD followed by 9 days of DD. **(B)** Percentage of rhythmic
478 flies are plotted for control (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts}), and (UAS Cas9; Pdf-g),* and
479 experimental (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts} > Cas9; Pdf-g)* males (left, n = 85 (*Pdf-RFP, Pdf-*
480 *Gal4; tub-Gal80^{ts}*), n = 89 (*UAS Cas9; Pdf-g*), n = 106 (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts} > Cas9;*
481 *Pdf-g*) and females (right, n = 79 (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts}*), n = 78 (*UAS Cas9; Pdf-g*), n
482 = 85 (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts} > Cas9; Pdf-g).* Error bars are SEM values plotted across 3
483 replicate experiments. **(C)** Free-running period of control (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts} and*

484 *UAS Cas9; Pdf-g)* and experimental (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts} > Cas9; Pdf-g)* males (left)
485 and females (right) calculated using the Chi-square periodogram are plotted. **(D)** Rhythmic power
486 of control (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts} and UAS Cas9; Pdf-g)* and experimental (*Pdf-RFP,*
487 *Pdf-Gal4; tub-Gal80^{ts} > Cas9; Pdf-g)* males (left) and females (right) calculated using the Chi-
488 square periodogram are plotted **(E)** The difference in rhythmic power of experimental males and
489 females from their respective controls are plotted. Flies were kept at 28°C throughout development
490 and as adults. The activity-rest behavior was recorded at 28°C. Free-running period and rhythmic
491 power values are calculated using Chi square periodogram in CLOCKLAB for a period of 7 days.
492 Statistical comparisons were performed between the control and experimental flies for both sexes
493 using the Kruskal-Wallis ANOVA followed by the Dunn's multiple comparisons test. The box plots
494 extend from the 25th to 75th percentile, with whiskers extending from the smallest to the largest
495 value, and each point representing data from a single fly. Combined data from at least 3 replicate
496 experiments are plotted.
497
498 **Figure 4: Speeding up the clock in M-cells leads to sexually dimorphic phenotypes. (A)**
499 Depiction of the adult *Drosophila* brain hemisphere indicating the clock cell subsets (colored)
500 having a faster running molecular clock. **(B)** Average activity plots of control (*Pdf-Gal4*) and
501 (UAS-*DBT^{s}*) and experimental (*Pdf > DBT^{s}*) flies are plotted for males (left) and females (right).
502 The plots are averaged over flies and days for a period of 3 days under LD 12:12. **(C)** The phase
503 of the morning peak of activity under LD 12:12 is plotted for control (*Pdf-Gal4*), (UAS-*DBT^{s}*) and
504 experimental (*Pdf >DBT^{s}*) males (left, n = 116 (*Pdf-Gal4*), n = 120 (UAS-*DBT^{s}*), n = 88 (*Pdf >*
505 *DBT^{s}*)) and females (right, n = 85 (*Pdf-Gal4*), n = 109 (UAS-*DBT^{s}*), n = 73 (*Pdf > DBT^{s}*)). **(D)**
506 Percentage of rhythmic flies are plotted for controls (*Pdf-Gal4), (UAS-DBT^{s}),* and experimental
507 (*Pdf > DBT^{s})* males (left, n = 90 (*Pdf-Gal4*), n = 108 (*UAS-DBT^{s}*), n = 93 (*Pdf > DBT^{s}*) and females
508 (right, n = 66 (*Pdf-Gal4*), n = 83 (*UAS-DBT^{s}*), n = 70 (*Pdf > DBT^{s}).* Error bars are SEM values
509 plotted across 3 replicate experiments. **(E)** Representative actograms of control (UAS-*DBT^{s}*) and
510 experimental (*Pdf > DBT^{s}*) males (left) and females (right) plotted for 6 days of LD followed by
511 10 days of DD. **(F)** Free-running period of control (*Pdf-Gal4), (UAS-DBT^{s})* and experimental (*Pdf*
512 *> DBT^{s})* males (left) and females (right) calculated using the Chi-square periodogram are plotted.
513 **(G)** Rhythmic power of control (*Pdf-Gal4), (UAS-DBT^{s})* and experimental (*Pdf > DBT^{s})* males
514 (left) and females (right) calculated using the Chi-square periodogram are plotted. **(H)** The

515 difference in rhythmic power of experimental males and females from their respective controls are
516 plotted. Free-running period and rhythmic power values are calculated using Chi-square
517 periodogram in Clocklab for a period of 7 days. Activity plots and phase of the M-peak were
518 calculated using PHASE. Statistical comparisons were performed between the control and
519 experimental flies for both sexes using the Kruskal-Wallis ANOVA followed by the Dunn's
520 multiple comparisons test. The box plots extend from the 25th to 75th percentile, with whiskers
521 extending from the smallest to the largest value, and each point representing data from a single fly.
522 Combined data from at least 3 replicate experiments are plotted.
523
524 **Figure 5: Speeding up the clock in a E-cell subset leads to a more advanced evening peak in**
525 **females. (A)** Depiction of the adult *Drosophila* brain hemisphere indicating the clock cell subsets
526 (colored) having a faster running molecular clock. **(B)** Average activity plots of control (*MB122B-*
527 *Gal4*) and (*UAS-DBT^s*) and experimental (*MB122B > DBT^s*) flies are plotted for males (left) and
528 females (right). The plots are averaged over flies and days for a period of 3 days under LD 12:12.
529 **(C)** Phase of the evening peak of activity under LD 12:12 for controls (*MB122B-Gal4*) and (*UAS-*
530 *DBT^s*) and experimental (*MB122B > DBT^s*) males (left, n = 92 (*MB122B-Gal4*), n = 93 (*UAS-*
531 *DBT^s*), n = 93 (*MB122B > DBT^s*)) and females (right, n = 85 (*MB122B-Gal4*), n = 87 (*UAS-DBT^s*),
532 n = 89 (*MB122B > DBT^s*)). **(D)** The difference in the phase of E-peak of experimental males and
533 females from their respective controls are plotted. **(E)** Free-running period of control (*MB122B-*
534 *Gal4* and *UAS-DBT^s)* and experimental (*MB122B > DBT^s)* males (left) and females (right)
535 calculated using the Chi-square periodogram are plotted. **(F)** Rhythmic power of control
536 (*MB122B-Gal4 and UAS-DBT^s)* and experimental (*MB122B > DBT^s)* males (left) and females
537 (right) calculated using the Chi-square periodogram are plotted. Free-running period and rhythmic
538 power values are calculated using Chi-square periodogram in CLOCKLAB for a period of 7 days.
539 Activity plots and phase of the M-peak were calculated using PHASE. Statistical comparisons
540 were performed between the control and experimental flies for both sexes using the Kruskal-Wallis
541 ANOVA followed by the Dunn's multiple comparisons test. The box plots extend from the 25th to
542 75th percentile, with whiskers extending from the smallest to the largest value, and each point
543 representing data from a single fly. Combined data from at least 3 replicate experiments are plotted.
544
545

546 **Supplementary figures**

547 **Figure S1: PDF rhythmic accumulation is similar in male and female brains. (Related to**
548 **Figure 1.) (A)** Representative confocal images of control (w^{1118}) (top) and experimental (Pdf^{01})
549 male (middle) and female (bottom) flies stained with PDF antibodies. **(B)** Scatterplots of PDF
550 staining intensities in s-LN_v dorsal projections of both male and female flies plotted at different
551 time-points over a 24-hr cycle on DD day3. Each dot represents the mean PDF intensity value
552 averaged over both the hemispheres of one brain. The cyan and pink lines are the best fit Cosine
553 curves from the parameters that were extracted using COSINOR analysis. See Table 2 for more
554 details. **(C)** Amplitude values of PDF oscillation obtained from COSINOR curve fits are plotted
555 for male and female flies. Error bars are 95% CI values calculated from the standard error obtained
556 from COSINOR analysis. Overlapping error bars indicate that amplitude values of males and
557 females are not significantly different from controls. COSINOR analysis was implemented using
558 the CATCosinor function from the CATkit package written for R [87]. n > 7 brain samples.

559

560 **Figure S2: CRISPR-Cas9 mediated *Pdf* mutagenesis has similar effects on male and female**
561 **sleep. (Related to Figure 3.) (A)** Average sleep plots under LD 12:12 of control (*Pdf-RFP, Pdf-*
562 *Gal4; tub-Gal80^{ts} and UAS Cas9; Pdf-g)* and experimental (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts} >*
563 *Cas9; Pdf-g)* males (left) and females (right) are plotted. The plots are averaged over flies and days
564 for a period of 3 days under LD 12:12 at 28°C. **(B)** Total sleep values under LD are plotted for
565 control (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts} and UAS Cas9; Pdf-g)* and experimental (*Pdf-RFP, Pdf-*
566 *Gal4; tub-Gal80^{ts} > Cas9; Pdf-g)* males (left) and females (right). **(C)** Average sleep plots of flies
567 over 7 days in DD are plotted for male and female control (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts} and*
568 *UAS Cas9; Pdf-g*) and experimental (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts} > Cas9; Pdf-g*) flies. **(D)**
569 Total sleep values under DD are plotted for control (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts} and UAS*
570 *Cas9; Pdf-g)* and experimental (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts} > Cas9; Pdf-g)* males (left) and
571 females (right). **(E)** The difference in the total DD sleep values of experimental males and females
572 from their respective controls are plotted.

573

574 **Figure S3: CRISPR-Cas9 mediated *Pdf* mutagenesis has more pronounced effects on male**
575 **circadian behavior at 25C. (Related to Figure 3.) (A)** Percentage of rhythmic flies are plotted
576 for control (*Pdf-Gal4), and (UAS Cas9; Pdf-g),* and experimental (*Pdf > Cas9; Pdf-g)* males (left,

577 *n* = 36 (*Pdf-Gal4*), *n* = 49 (*UAS Cas9; Pdf-g*), *n* = 52 (*Pdf > Cas9; Pdf-g*) and females (right, *n* =
578 33 (*Pdf-Gal4*), *n* = 46 (*UAS Cas9; Pdf-g*), *n* = 35 (*Pdf > Cas9; Pdf-g).* Error bars are SEM values
579 plotted across 2 replicate experiments. **(B)** Free-running period of control (*Pdf-Gal4 and UAS*
580 *Cas9; Pdf-g)* and experimental (*Pdf > Cas9; Pdf-g)* males (left) and females (right) calculated
581 using the Chi-square periodogram are plotted. **(C)** Rhythmic power of control (*Pdf-Gal4 and UAS*
582 *Cas9; Pdf-g)* and experimental (*Pdf > Cas9; Pdf-g)* males (left) and females (right) calculated
583 using the Chi-square periodogram are plotted. **(D)** The difference in the rhythmic power values of
584 experimental males and females from their respective controls are plotted.
585
586 **Figure S4: Adult specific CRISPR-Cas9 mediated *Pdf* mutagenesis has more pronounced**
587 **effects on male circadian behavior. (Related to Figure 3.) (A)** Representative confocal images
588 of control (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts}*) (top) and experimental (*Pdf-RFP, Pdf-Gal4; tub-*
589 *Gal80^{ts} > Cas9; Pdf-g*) male (middle) and female (bottom) flies stained with RFP and PDF
590 antibodies. The experimental flies show significant reduction in PDF levels but no misrouting of
591 the s-LN$_v$ dorsal termini. **(B)** Quantification of PDF intensity from s-LN$_v$ cell bodies in control
592 (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts}*) and experimental (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts} > Cas9; Pdf-*
593 *g*) flies are plotted for males (left) and females (right). Data points represent intensity from a single
594 brain sample. *n* > 10 brains for all genotypes. **(C)** The difference in PDF intensity values of
595 experimental males and females from their respective controls are plotted. **(D)** Percentage of
596 rhythmic flies are plotted for control (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts}), and (UAS Cas9; Pdf-g),*
597 and experimental (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts} > Cas9; Pdf-g)* males (left, *n* = 103 (*Pdf-RFP,*
598 *Pdf-Gal4; tub-Gal80^{ts}*), *n* = 109 (*UAS Cas9; Pdf-g*), *n* = 115 (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts} >*
599 *Cas9; Pdf-g*) and females (right, *n* = 96 (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts}*), *n* = 90 (*UAS Cas9; Pdf-*
600 *g*), *n* = 119 (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts} > Cas9; Pdf-g).* Error bars are SEM values plotted
601 across 3 replicate experiments. **(E)** Free-running period of control (*Pdf-RFP, Pdf-Gal4; tub-*
602 *Gal80^{ts} and UAS Cas9; Pdf-g)* and experimental (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts} > Cas9; Pdf-g)*
603 males (left) and females (right) calculated using the Chi-square periodogram are plotted. **(F)**
604 Rhythmic power of control (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts} and UAS Cas9; Pdf-g)* and
605 experimental (*Pdf-RFP, Pdf-Gal4; tub-Gal80^{ts} > Cas9; Pdf-g)* males (left) and females (right)
606 calculated using the Chi-square periodogram are plotted. Flies were raised at 18°C until eclosion
607 and were then transferred to 28°C as adults. Adult flies were kept at 28°C for a period of 7 days

before recording activity-rest behavior at 28°C. Free-running period and rhythmic power values are calculated using Chi square periodogram in CLOCKLAB for a period of 7 days. Statistical comparisons were performed between the control and experimental flies for both sexes using the Kruskal-Wallis ANOVA followed by the Dunn's multiple comparisons test. The box plots extend from the 25th to 75th percentile, with whiskers extending from the smallest to the largest value, and each point representing data from a single fly. Combined data from at least 3 replicate experiments are plotted.

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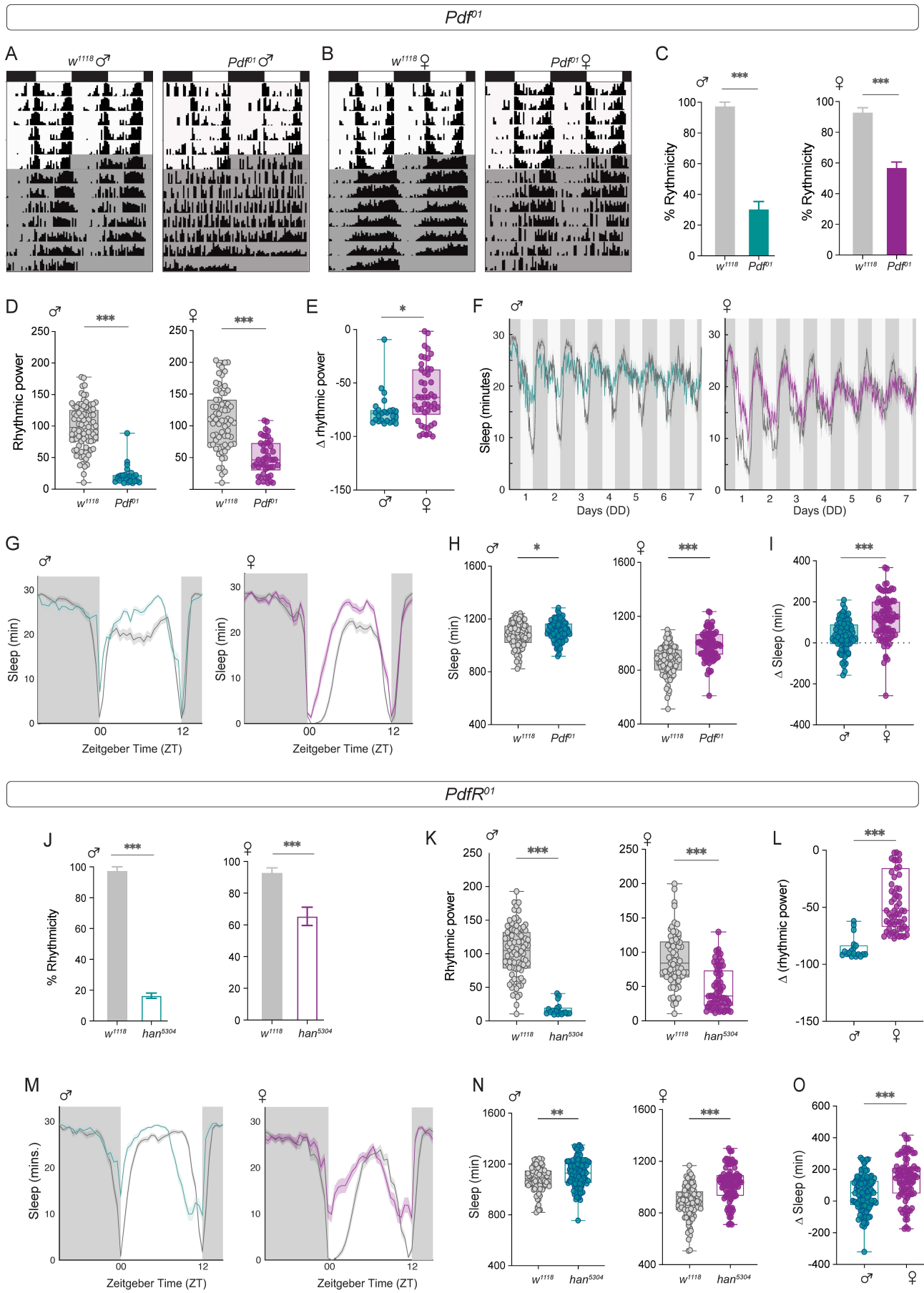
Figure 1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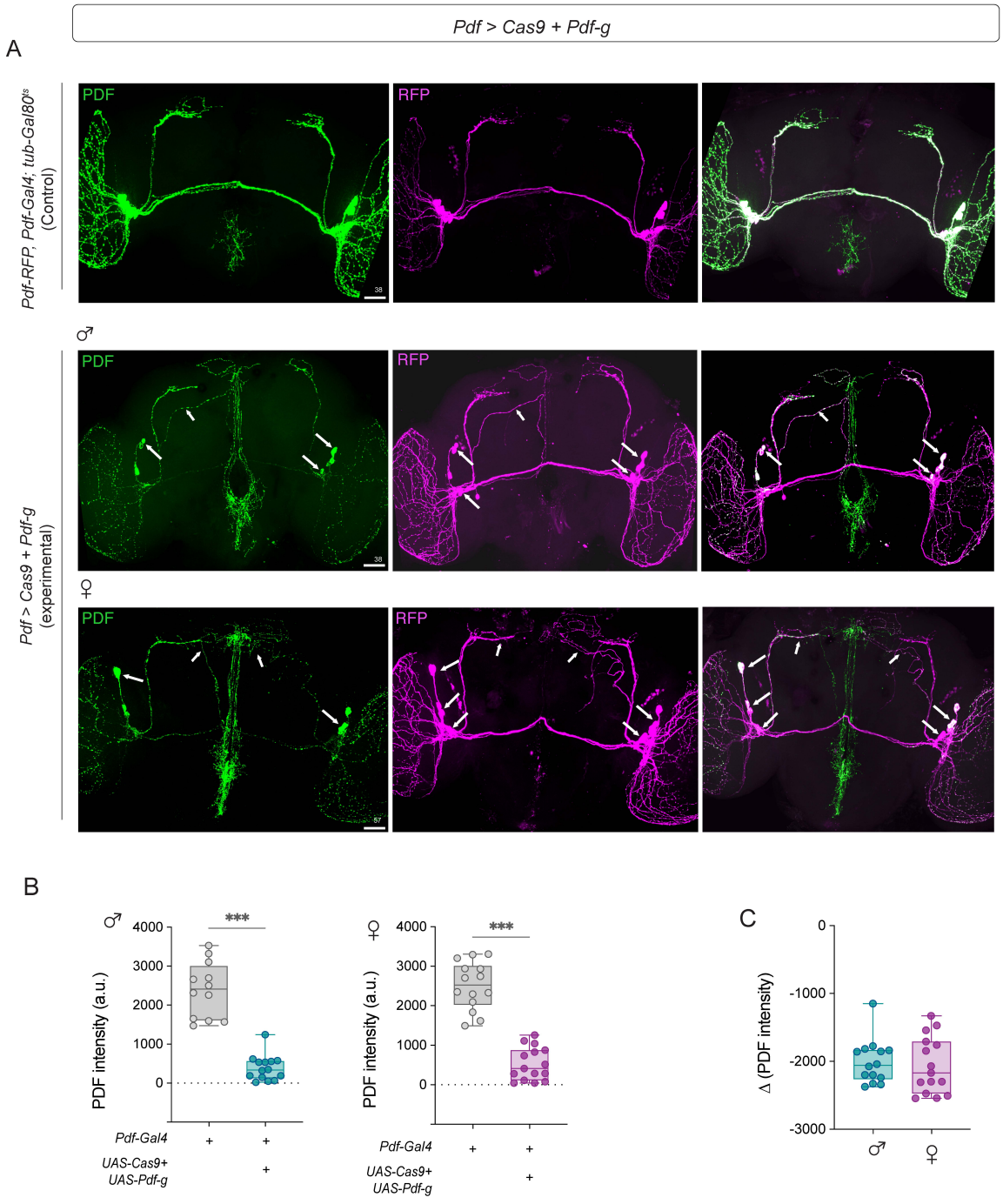
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Pdf > Cas9 + Pdf-g (experimental)
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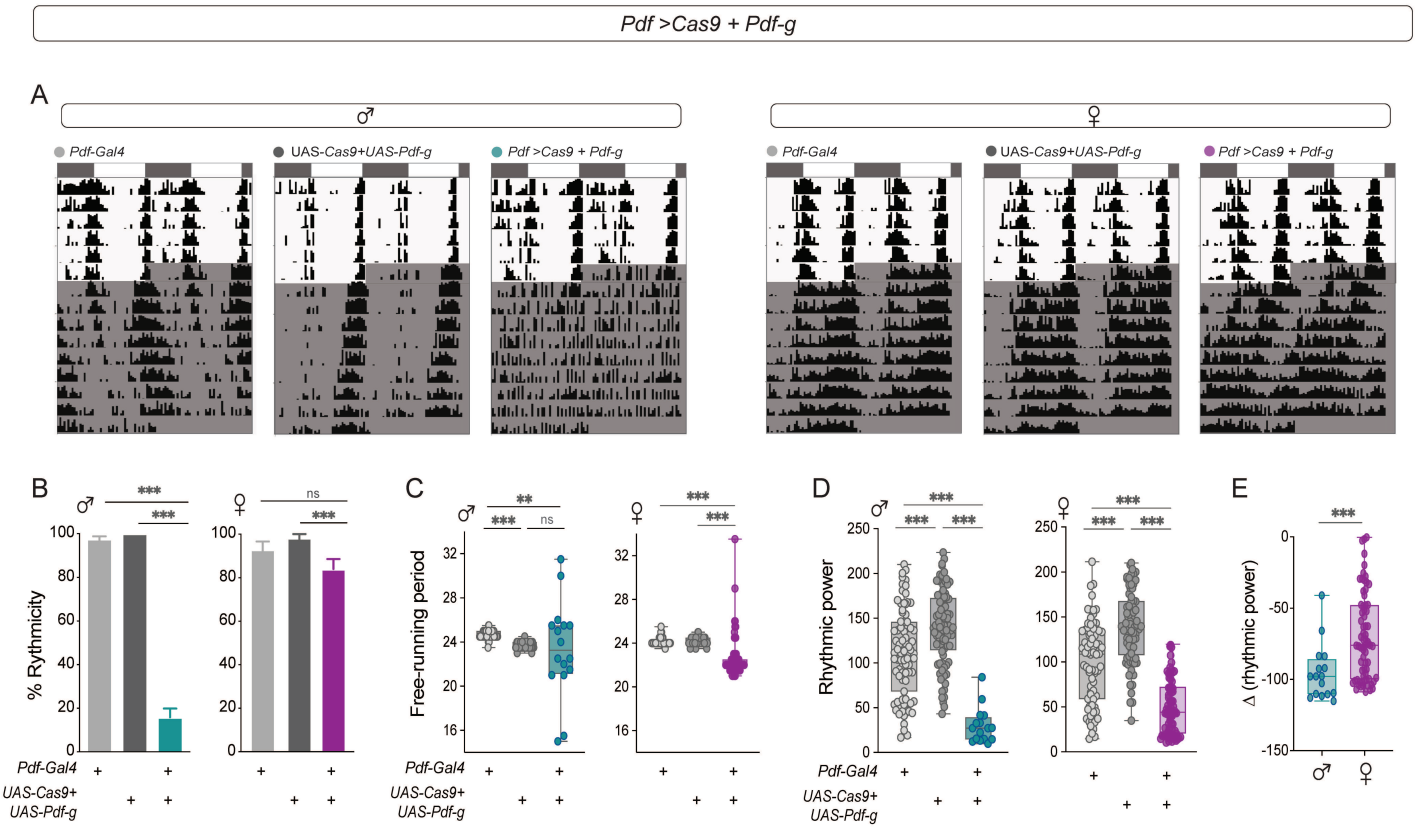
Figure 3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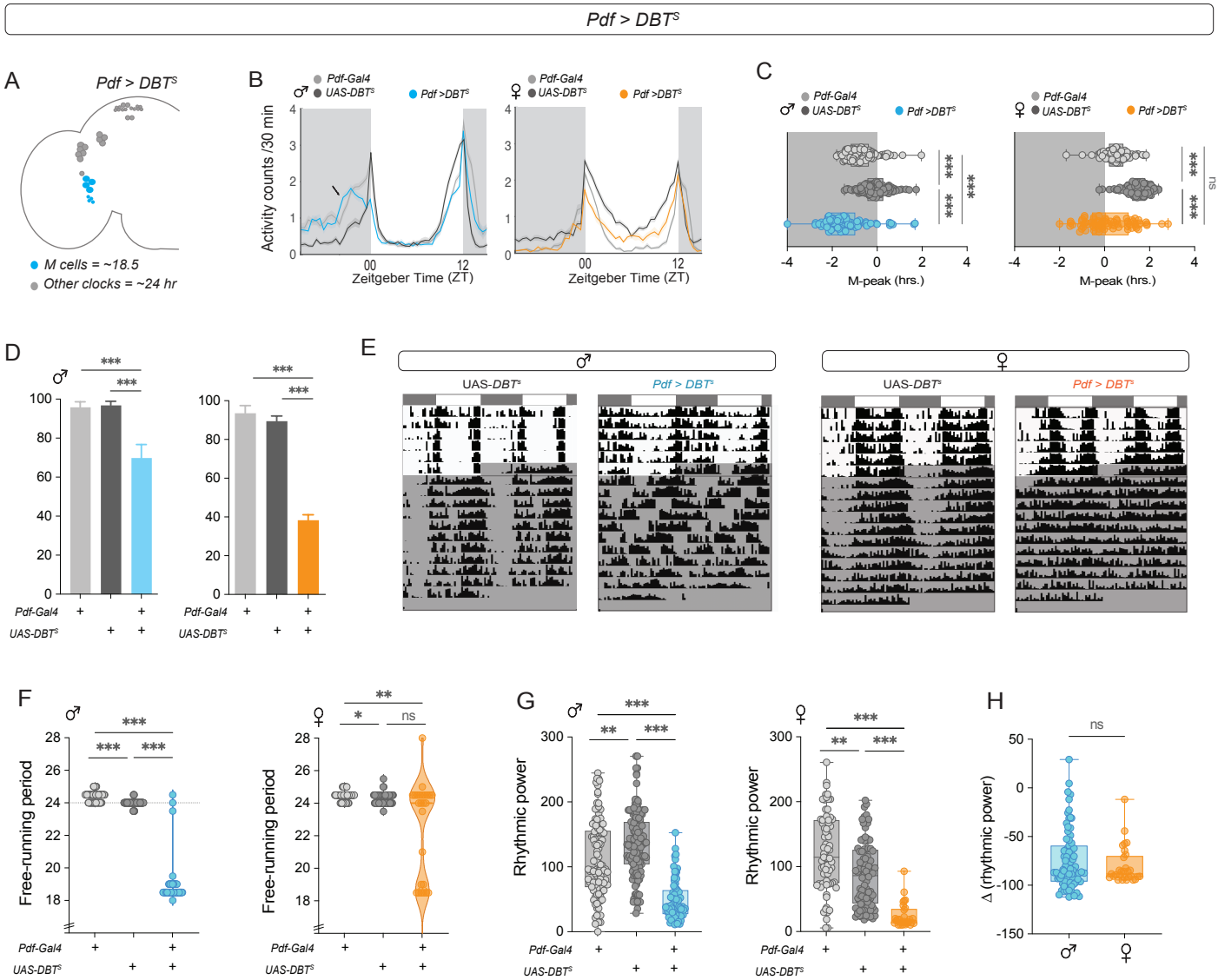
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Other clocks = ~24 hr
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UAS-DBTS
Pdf >DBTS
Activity counts /30 min
Zeitgeber Time (ZT)
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M-peak (hrs.)
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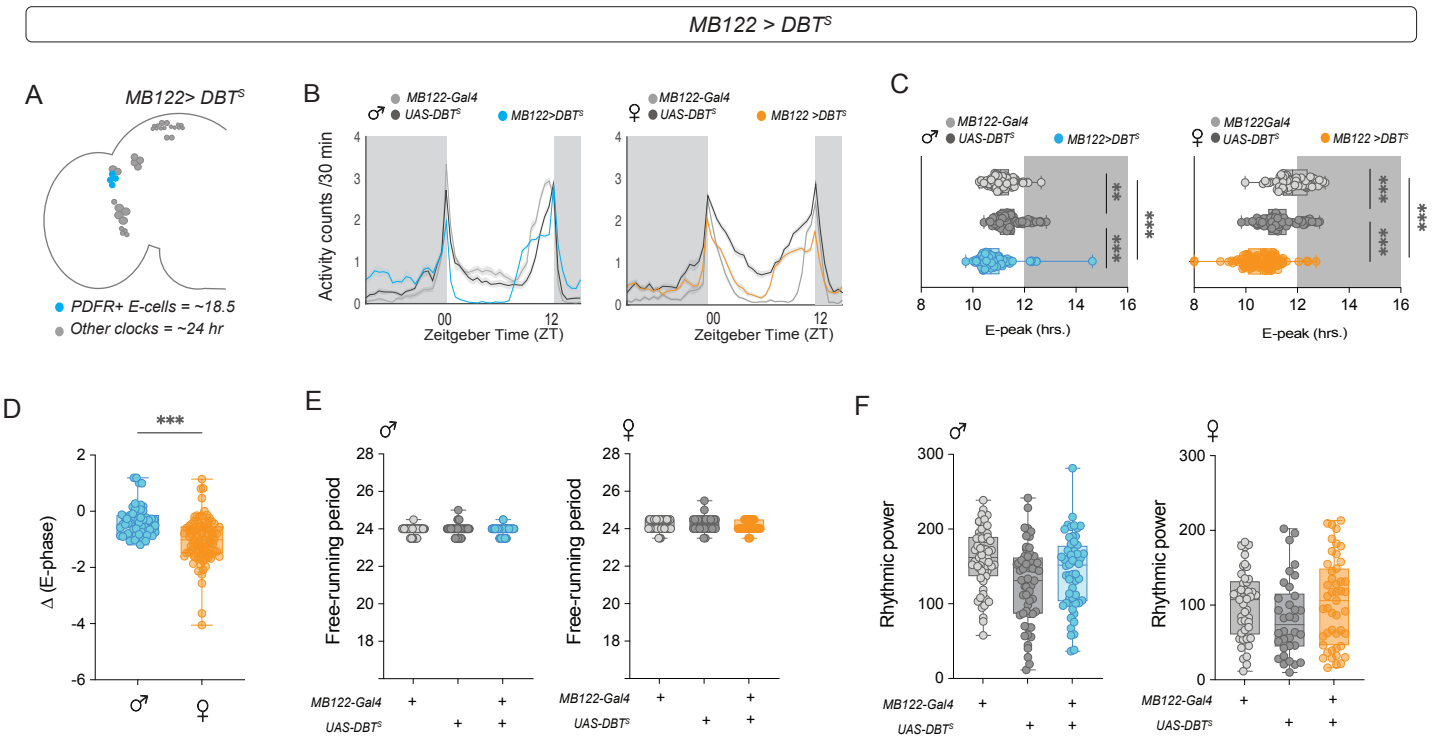
Figure 5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Other clocks = ~24 hr
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Activity counts /30 min
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Rhythmic power

Figure S1

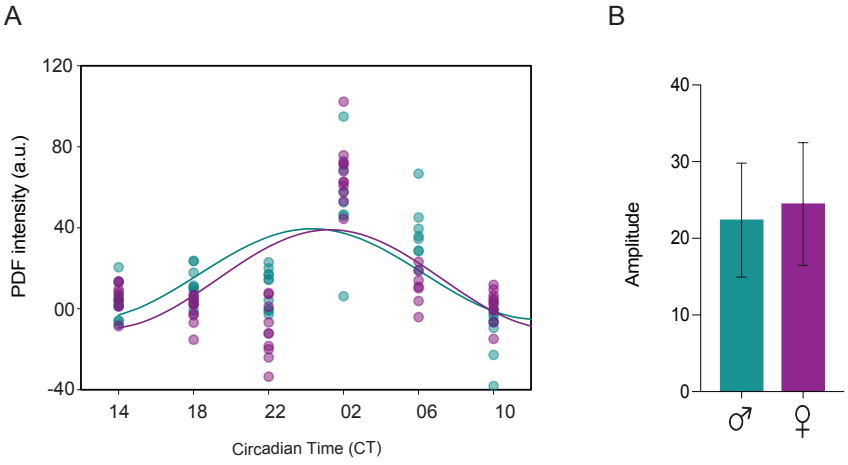
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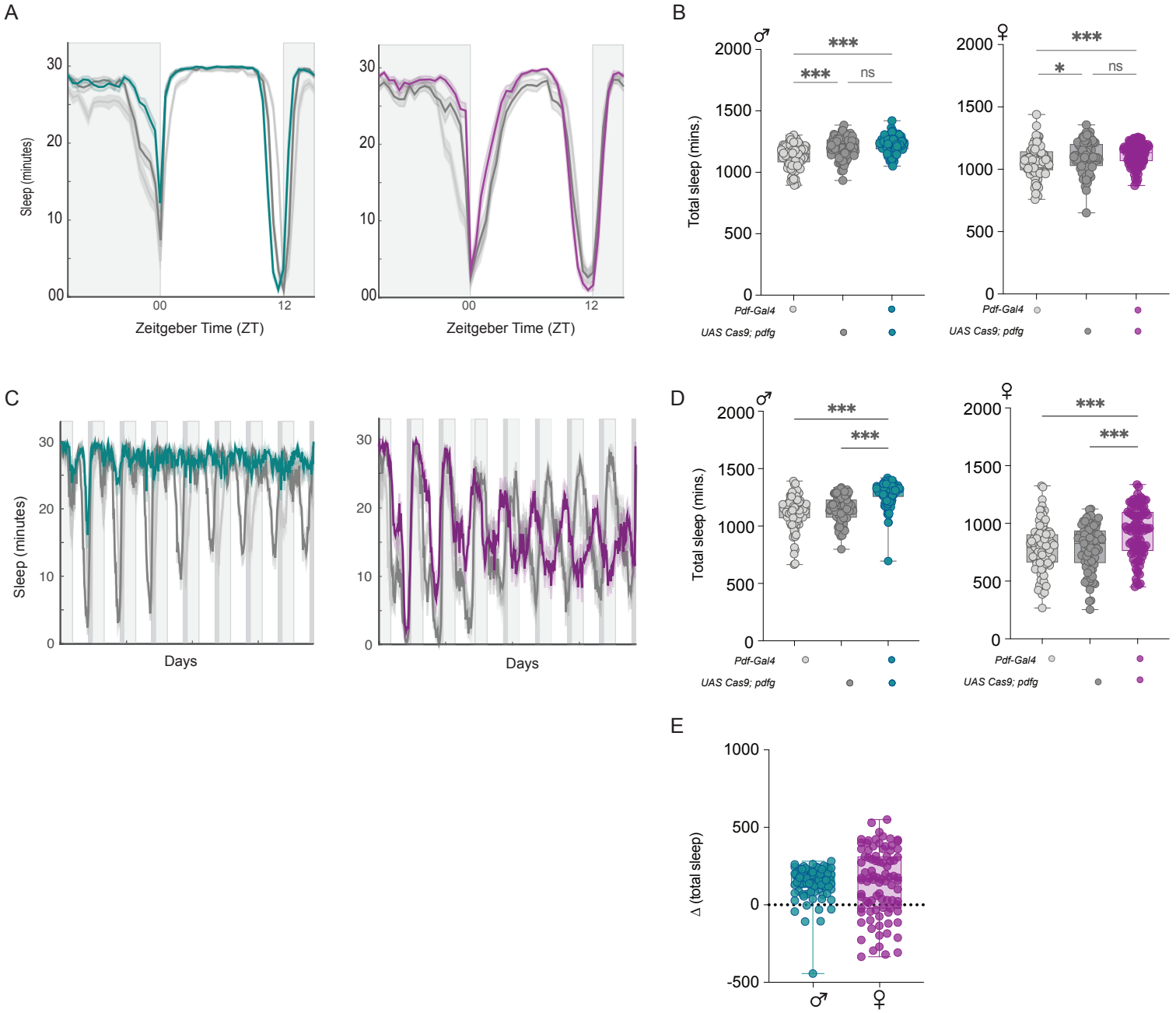
Figure S2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Sleep (minutes)
Zeitgeber Time (ZT)
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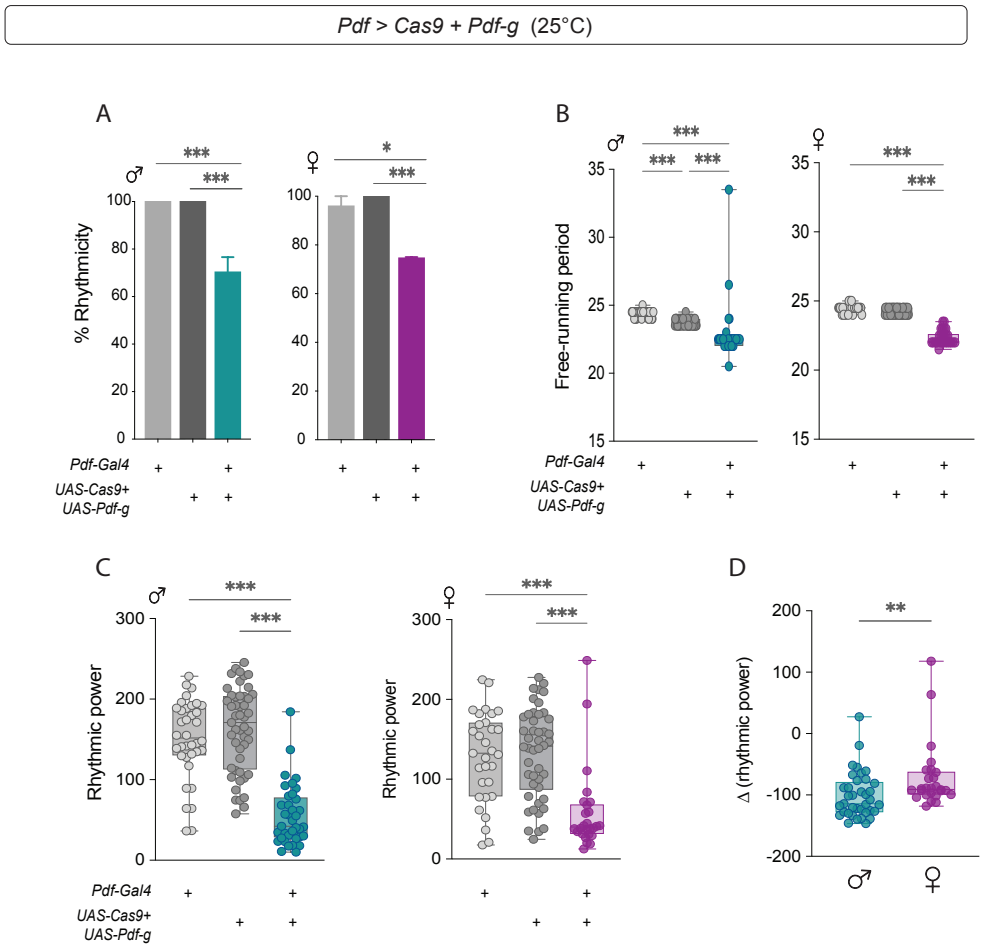
Δ (total sleep)

Figure S3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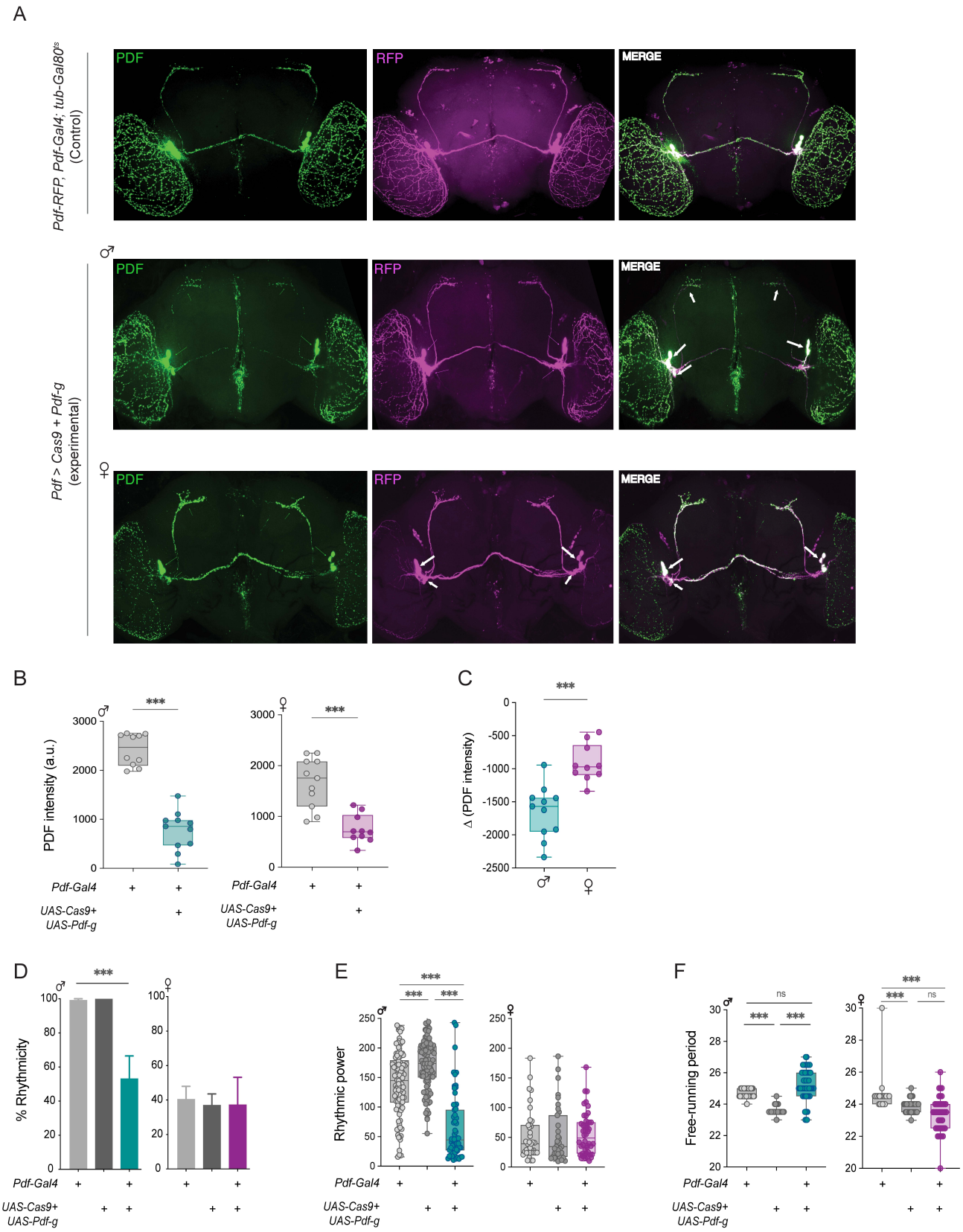
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