

1 **Effects of food availability cycles on phase and period of activity/rest rhythm in**

2 ***Drosophila melanogaster***

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9 Running title: *Food availability cycles do not entrain locomotor activity rhythm.*

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23 **Abstract**

24 Foraging and feeding are indispensable for survival and their timing depends not only on the  
25 metabolic state of the animal but also on the availability of food resources in their  
26 environment. Since both these aspects are subject to change over time, these behaviours  
27 exhibit rhythmicity in occurrence. As the locomotor activity of an organism is related to its  
28 disposition to acquire food, and peak feeding in fruit flies has been shown to occur at a  
29 particular time of the day, we asked if cyclic food availability can entrain their rhythmic  
30 activity. By subjecting flies to cyclic food availability *i.e.*, feeding/starvation (FS) cycles, we  
31 provided food cues contrasting to the preferred activity times and observed if this imposed  
32 cycling in food availability could entrain the activity/rest rhythm. We found that phase  
33 control, which is a property integral to entrainment, was not achieved despite increasing  
34 starvation duration of FS cycles (FS12:12, FS10:14 and FS8:16). We also found that flies  
35 subjected to T21 and T26 FS cycles were unable to match period of the activity rhythm to  
36 short or long T-cycles. Taken together these results show that external food availability  
37 cycles do not entrain the activity/rest rhythm of fruit flies. However, we find that starvation  
38 induced hyperactivity causes masking which results in phase changes. Additionally, T-cycle  
39 experiments resulted in minor period changes during FS treatment. These findings highlight  
40 that food cyclicality by itself may not be a potent zeitgeber but may act in unison with other  
41 abiotic factors like light and temperature to help flies time their activity appropriately.

42 **Key words:** Circadian clock, food entrainment, zeitgeber, locomotor activity rhythm,  
43 Feeding: Starvation cycles, masking, starvation induced hyperactivity, *Drosophila*  
44 *melanogaster*

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## 47 INTRODUCTION

48 Circadian clocks integrate cues from the environment and temporally regulate physiological  
49 and behavioural programs to aid animals fulfill their daily needs by anticipating cyclic  
50 changes in their day-to-day environment and time their physiology accordingly (Dunlap,  
51 2003). This is achieved by the process of entrainment which is the ability of the clock to  
52 synchronize to cyclic cues in the environment. Abiotic factors that cycle with time of day  
53 such as light and temperature serve as time cues or "zeitgebers" to the clock (Pittendrigh,  
54 1960); (Johnson et al., 2003). Similarly, biotic factors such as food resources may also serve  
55 as time cues to the clock of many animals (Hau and Gwinner, 1996); (Sharma et al., 2000);  
56 (Frisch and Aschoff, 1987).

57 Food resources frequently undergo changes in quality and quantity. While these changes are  
58 more apparent over seasons, daily food availability may also change as is documented in the  
59 case of various plant-pollinator and prey-predator interactions (Bloch et al., 2017); (Kronfeld-  
60 Schor et al., 2017). The cyclicity of these interactions are made possible by the circadian  
61 clocks that regulate diverse aspects of pollination such as those involved in flower  
62 advertisements in plants (Overland, 1960); (Matile, 2006); (Fenske et al., 2015); (Yon et al.,  
63 2016) and foraging activities in pollinator species (Fenske et al., 2018). Activity of several  
64 insect species such as bees (Bloch et al., 2017), mosquitoes (reviewed in (Sougoufara et al.,  
65 2017)), bedbugs (Romero et al., 2010) (reviewed in (Barrozo et al., 2004)) have been shown  
66 to be influenced by food availability.

67 Restricted food access in rodent models under laboratory conditions invokes an anticipatory  
68 response in the form of an activity bout before food availability called the food anticipatory  
69 activity (FAA) (Richter, 1922). FAA occurs for as long as food is restricted and even at  
70 times when animals are not usually active. For example, when food is restricted to daytime,

71 FAA is observed during daytime which is otherwise a period of low activity in nocturnal  
72 animals (Mistlberger, 2011); (Carneiro and Araujo, 2012). FAA is not dependent on the  
73 canonical light entrainable oscillator (LEO) located in the Suprachiasmatic Nucleus (SCN)  
74 (Stephan et al., 1979), and is thought to be controlled by another clock which is termed Food  
75 entrainable oscillator (FEO) which has not yet been localized (Pendergast and Yamazaki,  
76 2018). Furthermore, it has been shown that feeding entrains a peripheral clock in the liver  
77 (Damiola et al., 2000). Therefore, food provided at unusual times of the day can disrupt the  
78 phase relationship between the LEO and the liver peripheral clock. Other than the occurrence  
79 of FAA, the overall activity/rest pattern of these animals remains largely unaffected by  
80 change in food availability when SCN is intact. However, in SCN-lesioned animals, FEO  
81 can completely entrain activity rhythms to food availability cycles (Pendergast and  
82 Yamazaki, 2018), suggesting that food can act as a secondary zeitgeber

83 In the fruit fly, *Drosophila melanogaster* a peripheral clock in olfactory receptor neurons of  
84 the antenna regulates a circadian rhythm in olfactory responses with a peak in the middle of  
85 the night (Krishnan et al., 1999); (Tanoue et al., 2004). Similarly, a diurnal rhythm in  
86 electrophysiological responses of the labellar gustatory receptor neurons (GRN) has been  
87 reported with a peak in the morning hours. The GRN clock also regulates a behavioural  
88 gustatory rhythm in proboscis extension reflex (PrER); an appetitive behaviour with a peak in  
89 the morning (Chatterjee et al., 2010). Fruit flies also feed rhythmically with a peak in the  
90 morning in light/dark cycles and early subjective day in constant conditions (Xu et al., 2008).  
91 All these rhythms in *Drosophila* have been shown to be controlled by peripheral clocks while  
92 the activity/rest rhythm is known to be regulated by the central clock neurons (Helfrich-  
93 Forster, 1998). However, whether and how food availability affects any of these rhythms is  
94 unknown.

95 An organism's active phase is the time when most of the resource gathering and energy  
96 requirements are likely to be fulfilled. Hence locomotion of most animals is a function of  
97 various drives such as foraging, feeding, mating, oviposition etc. It is imperative to bring  
98 congruence between the internal drive to feed and the availability of food resources in the  
99 environment. Circadian clocks could facilitate this by adjusting the active phase of the  
100 organism such that the animal performs foraging and feeding behaviours while food is likely  
101 to be available in the environment. Since being in an active state is closely tied to an  
102 organism's disposition to acquire food resources, we asked if changing the time of food  
103 availability can bring about changes in the activity patterns and affect the underlying clock in  
104 *D. melanogaster*. In this study, we test the hypothesis that food availability cycles can act as  
105 a zeitgeber in entraining the activity/rest rhythm of *Drosophila melanogaster* by imposing  
106 various types of feeding cycles.

## 107 **METHODS**

### 108 *Locomotor Activity Assay*

109 Locomotor activity rhythm of flies was recorded using the *Drosophila* Activity Monitor  
110 (DAM, Trikinetics, USA). 4–5-day old virgin male flies, unless mentioned otherwise were  
111 recorded in LD 12:12 with *ad libitum* food (standard cornmeal medium) at 25°C for 2-3 days  
112 following which Feeding: Starvation (FS) cycles were imposed in constant dark (DD). The  
113 period of starvation lasted for either 12, 14 or 16 h depending upon the regime. Experimental  
114 flies received standard cornmeal food during the 'feeding' phase of the cycle and were  
115 transferred into 2% agar during the 'starvation' phase. FS cycles were imposed for 7 days  
116 following which flies were shifted to DD *ad libitum* food (DD *ad lib*) conditions for the next  
117 7 days. Age matched flies that were transferred into fresh food tubes at the same time as

118 experimental flies served as disturbance controls. All transfers were conducted under far-red  
119 light illumination (>630nm) in DD.

120 For the phase-shifted FS cycle experiment, 5–6-day old flies were subjected to the first FS  
121 (FS1) for 5 days after which the second FS (FS2) was imposed by either advancing or  
122 delaying the food transfers by 6 h with respect to FS1 for a period of 7 days. Acrophase  
123 (calculated using Actogram J, (Schmid et al., 2011)) was used as a phase marker to obtain the  
124 phase of the rhythm during and after FS cycles in all the regimes.

125 For T26 and T21 cycles, 5–6-day old virgin male flies were recorded in LD 12:12 with *ad*  
126 *libitum* food (standard cornmeal medium) at 25°C for 5 days following which FS cycles were  
127 imposed in constant dark. A T26 FS cycle was imposed on these flies such that the flies  
128 experienced 13 h of food availability and 13 h of starvation. Similarly, a T21 FS cycle was  
129 imposed where flies experienced 10.5 h of food availability and 10.5 h of starvation. Age  
130 matched flies served as disturbance controls as previously described. Seven such cycles were  
131 imposed following which flies were shifted to DD *ad libitum* food conditions for the next 7  
132 days. A *chi-square* periodogram analysis was done (using ClockLab software, Actimetrics,  
133 Wilmette, IL, USA) to determine periodicity during T26 and T21 feeding regimes as well as  
134 during DD *ad libitum* phase. In all the assays, flies were reared under LD12:12 regime  
135 before the start of the assay and all experiments were conducted at 25°C.

### 136 ***Statistical analyses***

137 Daily acrophases were compared using repeated measures ANOVA with day as the repeated  
138 measure and treatment as the between-group factor. Mauchly's test for sphericity was  
139 performed on all the data sets and Greenhouse-Geisser corrections were applied when the  
140 assumption for sphericity was not met. The above tests were performed using IBM, SPSS  
141 Statistics for windows (version 26, 2019, IBM corp., Armonk, N.Y., USA). Multiple *post*

142 *hoc* pairwise comparisons were performed using *t*-tests with Bonferroni corrections. Activity  
143 levels during starvation for all the FS regimes were analysed similarly on IBM, SPSS. Inter-  
144 individual phase synchrony between controls and experimental flies was tested by measuring  
145 the degree of dispersion of mean phases averaged across last 3 days of the FS cycles.  
146 Wallraff Rank sum test for angular dispersion was performed on phase values (radians) using  
147 R core team (version 3.6.1, 2019, R: A language and environment for statistical computing. R  
148 Foundation for Statistical Computing, Vienna, Austria. URL <http://www.R-project.org/>).  $\Delta$   
149 phase – defined as change in phase on the first day of DD *ad lib* from the mean acrophase  
150 (last 3 days) during FS regime, was compared using one sample *t*-test against a reference  
151 constant 0. Additionally, two sample *t*-test was used to compare  $\Delta$  phases between controls  
152 and experimental flies. For T21 & T26 experiments, a Chi Square test for proportions was  
153 performed using GraphPad Prism (version 9.2.0 for Windows, GraphPad software, San  
154 Diego, California, USA, [www.graphpad.com](http://www.graphpad.com)) to compare proportions of flies exhibiting  
155 different periodicities. Change in period was tested using Mann-Whitney U test. All other  
156 analyses were performed on STATISTICA (version 7, 2004, StatSoft Inc, Tulsa, OK, USA).

## 157 **RESULTS**

158 In order to address if external food availability cycles or the Feeding: Starvation (FS) cycles  
159 entrain the activity/rest rhythm of *D. melanogaster*, we subjected flies to three different FS  
160 cycles of increasing starvation duration – namely, FS12:12 (12 h of feeding followed by 12 h  
161 of starvation, Fig 1A), FS10:14 (10 h of feeding followed by 14 h of starvation, Fig 1B) and  
162 FS8:16 (8 h of feeding followed by 16 h of starvation, Fig 1C). Activity/rest rhythms were  
163 recorded in LD12:12 at 25°C on *ad libitum* food for 2-3 days before shifting to one of the  
164 aforementioned FS regimes in constant dark (DD). In all three FS regimes, the feeding  
165 duration overlapped, partly or completely with daytime in the previous LD cycles. Following  
166 7-8 days of FS regime, flies were subjected to DD with *ad libitum* food (DD *ad lib*).

167 Disturbance control and experimental flies show startle bouts of activity when they are  
168 moved to new tubes (Fig 1A-C, left, arrows). While these startle bouts can be attributed to  
169 disturbance due to change of tubes, we expected to observe changes in the activity/rest  
170 rhythm because of FS cycles over and above the disturbance caused during the assay (Fig 1).  
171 For example, as expected from previous studies (Connolly, 1966), experimental flies show  
172 increased activity level during starvation compared to control flies (Fig 1A-C, right). To  
173 determine if FS cycles are indeed entraining the activity/rest rhythm we examined classical  
174 criteria of entrainment, namely – day-to-day phase stability, inter-individual phase  
175 synchrony, phase control and period matching with zeitgeber cycle ( $\tau = T$ ) (Moore-Ede et al.,  
176 1982). We used acrophase which is the radial centre of mass of activity (Diez-Noguera,  
177 2013) as the phase marker in all the experiments.

#### 178 *Day-to-day phases vary in flies subjected to FS cycles of 10:14 and 8:16*

179 We tested day-to-day stability of phases to determine stable phase relationship with zeitgeber  
180 cycles. To compare daily phases, we performed repeated measures ANOVA on acrophases  
181 of control and experimental flies during FS treatment with day as the repeated measure and  
182 treatment as a between-group fixed factor. We found that daily acrophases of flies subjected  
183 to FS12:12 were similar to those of control flies on all cycles. Further, acrophases of both  
184 groups on first two cycles were different from acrophases in subsequent cycles suggesting  
185 phase changes due to startle responses in both control and experimental flies (Fig 2A,  
186 repeated measures ANOVA, Greenhouse-Geisser  $\epsilon = 0.56$ ,  $F_{(3,34, 176.92)} = 8.93$ , main effect of  
187 day,  $p < 0.001$  followed by pair-wise  $t$ -tests with Bonferroni corrections for 21 comparisons).  
188 In FS10:14 regime, on day 1, controls show a significantly different phase compared to  
189 phases on subsequent cycles 2 and 3 suggesting that day 1 phase is affected by disturbance  
190 (Fig 2B, Greenhouse-Geisser  $\epsilon = 0.66$ ,  $F_{(3,98,222.99)} = 5.66$ , day  $\times$  treatment,  $p < 0.001$ ,



191 followed by pair-wise  $t$ -tests with Bonferroni corrections for 49 comparisons). However, it  
192 stabilizes within a day as phases from the 2nd to the 7th cycle remain unchanged.  
193 Experimental flies under FS10:14 show gradual changes in day-to-day phases. Acrophases in  
194 the first two cycles of the FS regime are significantly different from the last two cycles of the  
195 treatment (Fig 2B). Furthermore, we also observed that experimental flies have acrophases  
196 which are significantly different from controls in the first half of the treatment (cycles 1-3)  
197 whereas these acrophases start resembling acrophases of controls in the second half of the  
198 treatment (cycles 4-7). This suggests that imposing FS10:14 cycles result in transient phase  
199 changes which gradually disappear after a few cycles.

200 Similarly, phases of the experimental flies experiencing FS8:16, also change gradually across  
201 days with acrophases on the first and second cycles being significantly different from  
202 acrophase on last day of the treatment (8th cycle, Fig 2C, Greenhouse-Geisser  $\epsilon = 0.74$ ,  $F_{(5,16,237.45)} = 5.26$ , day  $\times$  treatment,  $p < 0.001$ , followed by pair-wise  $t$ -tests with Bonferroni  
203 corrections for 64 comparisons). On the other hand, control flies do not show any phase  
204 changes across all cycles. Additionally, similar to FS10:14, acrophases of FS8:16 flies are  
205 significantly different from controls from the beginning of the treatment (cycles 2-4) but start  
206 resembling acrophases of the controls towards the end of the treatment (cycles 5-8).  
207

208 Controls in all the regimes are affected by disturbance on the first day of FS cycle after which  
209 they show phase stability. On the other hand, phases of experimental flies are affected on the  
210 first few FS cycles which take longer to attain phases similar to the controls. Given that the  
211 internal period is not too different from 24 h (Table 1), it is difficult to ascertain whether this  
212 day-to-day phase stability in the latter half of FS treatment is due to free-running, masking or  
213 entrainment. Hence, we used other measures to differentiate between these possibilities.

214 ***Feeding: Starvation cycles do not increase inter-individual phase synchrony***

215 An entrained rhythm is phase-locked to the zeitgeber cycle, and this phase relationship is  
216 stable across multiple cycles and reproducible across individuals. This implies that in an  
217 entrained condition, individual flies will exhibit similar phases resulting in higher inter-  
218 individual synchrony. Here we examined the extent of phase dispersion within control and  
219 experimental fly groups during FS regimes. If FS cycles were entraining the activity/rest  
220 rhythm we would expect a smaller dispersion with greater consolidation of phases under  
221 entrained conditions. We find that the degree of dispersion of acrophases of flies in each of  
222 the experimental regimes of FS12:12 (Fig 3A, Wallraff rank sum test for angular dispersion,  
223 Kruskal-Wallis Chi Square = 2.32,  $df = 1$ ,  $p = 0.13$ ), FS10:14 (Fig 3C, Kruskal-Wallis Chi  
224 Square = 0.007,  $df = 1$ ,  $p = 0.93$ ) and FS8:16 (Fig 3E, Kruskal-Wallis Chi Square = 1.31,  $df =$   
225  $1$ ,  $p = 0.25$ ) was not statistically different from their respective disturbance controls  
226 suggesting that FS cycles are not efficient in synchronizing the phases of experimental flies.

227 Additionally, we asked whether abrupt shifts in food availability schedules may reveal any  
228 features that were not previously detectable. Two jetlag FS12:12 experiments were  
229 conducted, where the second set of FS cycles (FS2) were either phase delayed (Supp Fig 1A)  
230 or advanced (Supp Fig 1B) with respect to previous FS cycle (FS1). Similar to the previous  
231 FS12:12 (Fig 1), the activity rhythm of the experimental flies during FS1 and FS2 was  
232 similarly phased as their disturbance controls (Supp Fig 1). The degree of phase dispersion  
233 during the first FS cycle and second FS cycle was also not different in experimental flies as  
234 compared to their controls in both phase delay (Fig 4A, FS1- Kruskal-Wallis Chi Square =  
235  $0.9$ ,  $df = 1$ ,  $p = 0.77$ , FS2- Kruskal-Wallis Chi Square =  $1.67$ ,  $df = 1$ ,  $p = 0.2$ ) and advance  
236 conditions (Fig 4B, FS1- Kruskal-Wallis Chi Square =  $0.88$ ,  $df = 1$ ,  $p = 0.35$ , FS2- Kruskal-  
237 Wallis Chi Square =  $1.33$ ,  $df = 1$ ,  $p = 0.25$ ). These results suggest that FS cycles fail to  
238 increase inter-individual synchrony of activity rhythms.

239 ***Feeding: Starvation cycles of different starvation durations do not exert phase control on***  
240 ***activity/rest rhythm***

241 To test if the phase in constant conditions follows from the previously entrained phase (phase  
242 control), the change in acrophase ( $\Delta$  phase) was quantified by subtracting acrophase on the  
243 first day of DD *ad lib* from the mean acrophase (last 3 days) during FS regime. In case of  
244 phase control, the expectation is that  $\Delta$  phase will not be significantly different from zero.  
245 We found that the  $\Delta$  phase was significantly different from zero in both the controls as well  
246 as FS12:12 flies suggesting that the disturbance itself brings about some change in phase in  
247 both the groups (Fig 3B, left, experimental  $t_{(27)} = 5.73$ ,  $p < 0.001$ , One sample *t*-test against  
248 reference constant 0 and control  $t_{(26)} = 2.76$ ,  $p = 0.01$ ). However,  $\Delta$  phase in the experimental  
249 flies is significantly higher than the controls implying that the FS cycles fail to exert phase  
250 control (Fig 3B, left  $t_{(53)} = 2.45$ ,  $p = 0.018$ , Two sample *t*-test). Additionally, when two  
251 FS12:12 cycles were imposed consecutively (FS1 & FS2), either phase delayed (Fig 4C,  
252 Supp Fig 1A) or phase advanced (Fig 4D, Supp Fig 1B) with respect to one another, the  
253 phase of the activity/rest rhythm continued to follow from the previous entrained phase in  
254 LD12:12 irrespective of FS2 suggesting lack of phase control (Fig 4C, D, Supp Fig 1) even in  
255 these regimes. Here again,  $\Delta$  phase was significantly different from zero in experimental  
256 flies experiencing phase shifted FS cycles suggesting lack of phase control (Fig 4C,  $t_{(29)} =$   
257  $3.37$ ,  $p = 0.002$ , One sample *t*-test against reference constant 0 and Fig 4D,  $t_{(27)} = 3.29$ ,  $p =$   
258  $0.0028$ , One sample *t*-test against reference constant 0) as well as controls in the phase delay  
259 regime suggesting some disturbance related effects (Fig 4C,  $t_{(27)} = 2.88$ ,  $p = 0.007$ , One  
260 sample *t*-test against reference constant 0). Even though control flies of FS12:12 show  $\Delta$   
261 phase value which is significantly different from zero, we found that the PERIOD (PER)  
262 accumulation rhythm in the ventrolateral neurons (LN<sub>v</sub>) neurons of individual fly brains does

263 not undergo any change because of disturbance alone (*data not shown*) suggesting that  
264 disturbance does not affect the central clock.

265 When the duration of starvation was increased to 14 h in FS10:14, or to 16 h in FS8:16,  $\Delta$   
266 phase was significantly different from zero in the experimental flies but not in controls (Fig  
267 3D, left,  $t_{(28)} = 4.14$ ,  $p = 0.0002$ , One sample  $t$ -test against reference constant 0 and Fig 3F,  
268 left  $t_{(22)} = 3.6$ ,  $p = 0.0015$ , One sample  $t$ -test against reference constant 0). This suggests that  
269 lack of phase control persists in experimental flies despite increasing starvation. In addition,  
270  $\Delta$  phase of experimental flies experiencing FS10:14 and FS8:16 was significantly higher than  
271 the controls (Fig 3D, left,  $t_{(56)} = 3.34$ ,  $p = 0.0015$ , Two sample  $t$ -test and Fig 3F, left,  $t_{(46)} =$   
272  $2.035$ ,  $p = 0.0475$ , Two sample  $t$ -test). In FS10:14 the mean phase during the treatment is  
273 marginally different between the control and experimental flies (Fig 3D, right,  $t_{(56)} = 2.0007$ ,  
274  $p = 0.0502$ , Two sample  $t$ -test). The change in mean phase during FS can be attributed to  
275 acrophases affected by starvation induced hyperactivity (SIH, Fig 1B, right).

276 In all the FS regimes tested, we found that  $\Delta$  phase was different from zero in all  
277 experimental flies (and also in FS12:12 controls). This suggests that the stable phase attained  
278 during the last three FS cycles changed quickly on the first day of DD *ad lib*, which is  
279 characteristic of masking. SIH was observed on all days among experimental flies which  
280 may influence acrophases resulting in masking (Fig 1, right, Supp Fig 3). Previously we had  
281 seen that FS regime induces some phase changes that gradually return to phase values similar  
282 to controls during the latter half of the regime which may reflect free running phases in flies  
283 experiencing FS. This suggests that phases during the FS cycles were possibly intermediary  
284 between the internal clock and masking components. Overall, lack of phase control and  
285 lower inter-individual synchrony suggests that FS cycles may not bring about stable  
286 entrainment in activity/rest rhythms in *Drosophila melanogaster*.

287 Since female flies feed more compared to males (Wong et al., 2009), we asked if FS cycles  
288 can entrain activity/rest rhythm in female flies. We found similar results when female flies  
289 were subjected to FS8:16 wherein most flies showed excessive activity in the starvation  
290 window (Supp Fig 2A). Average phase was significantly different from the controls during  
291 FS8:16 which immediately reverted to pre-FS phase after the FS treatment (DD *ad lib*) (Supp  
292 Fig 2B, repeated measures ANOVA with day as the repeated measure and treatment as  
293 between-group fixed factor was performed (main effect of treatment  $F_{(1, 47)} = 27.51, p <$   
294  $0.001$ ); C-centre  $t_{(47)} = 2.66, p = 0.0105$ , Two sample *t*-test). Additionally, we found that  
295 there was no phase control - as  $\Delta$  phase was significantly different from zero (Supp Fig 2C,  
296 left,  $t_{(24)} = 4.96, p < 0.001$ , One sample *t*-test against reference constant 0) nor was there  
297 inter-individual synchrony (Supp Fig 2C-right, Wallraff rank sum test for angular dispersion).  
298 Altogether, these results indicate that activity rhythms of both males and females do not  
299 entrain to FS cycles.

### 300 ***Starvation Induced hyperactivity is not sustained across FS cycles***

301 Starvation has been shown to result in excess activity levels which fall to the baseline levels  
302 after food has been provided (Connolly, 1966); (Yang et al., 2015). Interestingly, we  
303 observed that cyclic food availability for several cycles does not result in consistent  
304 hyperactivity across all cycles. Flies that experienced 12 h of starvation per day for 7  
305 consecutive days, showed activity levels comparable to the controls in the 12 h starvation  
306 window each day. However, activity levels on the first two days were higher as compared to  
307 other days (Supp Fig 3A, repeated measures ANOVA, Greenhouse-Geisser  $\epsilon = 0.44, F_{(2.67,$   
308  $149.3)} = 28.39$ , main effect of day,  $p < 0.001$ , followed by pair-wise *t*-tests with Bonferroni  
309 corrections for 21 comparisons). When flies were subjected to FS10:14, they showed an  
310 immediate increase in activity in response to lack of food (Fig 1B right, Supp Fig 3B). The  
311 activity levels were higher than the controls in the first 3 cycles of the treatment, after which

312 they were comparable to the controls (Supp Fig 3B, Greenhouse-Geisser  $\epsilon = 0.52$ ,  $F_{(3.13, 175.32)}$   
313  $= 3.72$ , day  $\times$  treatment,  $p = 0.012$ , followed by pair-wise  $t$ -tests with Bonferroni corrections  
314 for 49 comparisons). Interestingly, we found two types of behaviours among individuals  
315 when subjected to 16 h of starvation, 33.3% flies (7/23) showed excessive activity during  
316 starvation hours throughout the 8 days of FS 8:16 (type *a* flies, Fig 1C; centre) and 60.9 %  
317 flies, (14/23) appeared to show excessive activity during starvation only for the first few days  
318 after which the activity seemed to decrease (type *b* flies, Fig 1C; right). Day-to-day activity  
319 levels of FS8:16 flies (Supp Fig 3C, Greenhouse-Geisser  $\epsilon = 0.29$ ,  $F_{(2.05, 108.54)} = 6.99$ , day  $\times$   
320 treatment,  $p = 0.001$ , followed by pair-wise  $t$ -tests with Bonferroni corrections for 64  
321 comparisons) showed reduction in activity levels after 6 days (Fig 1C right, Supp 3C). This  
322 shows that phases during FS are masked as a result of hyperactivity occurring in response to  
323 starvation.

324 ***T26 and T21 Feeding: Starvation cycles do not synchronize the activity/rest rhythm of***  
325 ***Drosophila melanogaster***

326 We then assessed the third criterion for entrainment, i.e., period matching between  
327 activity/rest rhythm ( $\tau$ ) and external FS cycles (T). In the previous experiments given that T  
328 was 24 h, and  $\tau$  was also close to 24 h (Table 1) it was not possible to test this criterion using  
329 data from FS12:12, 10:14 and 8:16 experiments. We therefore imposed FS cycles whose  
330 periods were not 24 h, but also not very deviant from 24 h to account for a possibility of a  
331 food entrainable oscillator if present, having narrow limits of entrainment. We imposed  
332 either a 26 h or a 21 h FS regime and asked if this could result in period matching of the  
333 activity/rest rhythm to external FS cycles. We subjected the flies to T26 FS cycles wherein  
334 the flies were provided food for 13 hours and were starved for 13 hours (Fig 5A) in an  
335 otherwise aperiodic environment (DD, 25°C). A large majority (Fig 5B) of the experimental

336 flies appear to free run with a phase similar to the previous entrained phase in LD12:12  
337 despite being subjected to T26 FS regime. However, they also exhibited a masked  
338 component to the disturbance caused due to change of tubes (Fig 5A, green dashed line). A  
339 Chi square periodogram analysis during T26 FS revealed 2 significant periodicities; one that  
340 was close to 24 h (henceforth referred to as free running component) and another that was  
341 close to 26 h (Table 2, Fig 5B). Since both control and experimental flies exhibit this long  
342 period (~26 h) component, the same can be attributed to the physical disturbance experienced  
343 by both the controls and experimental flies (henceforth referred to as the masking  
344 component). Presence of two significant periodicities in a large proportion of experimental  
345 flies similar to control flies suggests that a T26 FS regime is unable to synchronise activity  
346 (Fig 5B, Chi-square test for proportions shows no difference in the two groups ( $p = 0.18$ , Chi-  
347 square = 3.22,  $df = 2$ ). We estimated the difference in free running component (~24h period)  
348 during FS ( ${}^{24}\tau_1$ , T26 FS) and after FS ( ${}^{24}\tau_2$ , DD *ad lib*) for each fly. Interestingly, we found  
349 that experimental flies exhibited approximately 23 minutes longer period during T26 FS  
350 regime. This difference was significantly greater than the controls suggesting that T26 FS  
351 indeed significantly lengthens the internal period of the flies, albeit to a small degree (Fig 5C,  
352  $U = 2388$ ,  $p < 0.001$ , Mann-Whitney U test, Table 2).

353 Like the T26 regime, we also subjected the flies to a T21 regime with 10.5 h of food  
354 availability followed by 10.5 h of starvation in DD 25°C. We found similar results wherein  
355 the experimental flies continued to free run from the previously entrained LD phase along  
356 with a masked response to the external food availability cycles (Fig 6A, green dashed line).  
357 Akin to T26, we found flies exhibiting 2 periodicities, a near 24 h component (free running  
358 component) and a near 21 h component (masked component) as estimated by Chi square  
359 periodogram (Fig 6B). A higher proportion of T21 FS flies show only 21 h periodicity as  
360 compared to controls, (Chi-square test for proportions shows a significant difference  $p <$

361 0.001, Chi-square = 15.37,  $df = 2$ ) yet majority of the experimental flies still show both 21h  
362 and 24h periodicities. This further provided evidence for inability of the external T21 FS  
363 cycle to synchronize the activity/rest rhythm because of incomplete period matching.  
364 Moreover, like the T26 regime, T21 regime also affects the intrinsic period during the  
365 treatment. We estimated the difference in free running period during FS ( $^{24}\tau_1$ , T21 FS) and  
366 after FS ( $^{24}\tau_2$ , DD *ad lib*) for each fly and we found that experimental flies exhibited  
367 approximately 24 minutes shorter period during T21 FS regime. This difference was  
368 significantly lower than the controls suggesting that T21 FS significantly shortens the  
369 intrinsic period of the experimental flies (Fig 6C,  $U = 2389$ ,  $p < 0.001$ , Mann-Whitney U test,  
370 Table 3). Overall, inability to match the intrinsic period to T cycles suggests that FS cycles  
371 are inefficient in synchronizing the activity/rest rhythm. Thus, another criterion for  
372 entrainment (period matching:  $\tau = T$ ) is not met under conditions where food availability is  
373 cyclic. These results taken together, suggest that FS cycles do not entrain the activity/rest  
374 rhythms controlled by the central clock in *D. melanogaster*.

## 375 **DISCUSSION**

376 We examined if cyclic food availability can act as an entraining cue for the clock in fruit fly  
377 *Drosophila melanogaster*. We assessed three criteria for entrainment - day-to-day phase  
378 stability, phase control and period matching. We found that subjecting flies to FS cycles of  
379 different durations of starvation does not result in inter-individual synchrony and phase  
380 control. These results build on the findings of a previous study in which subjecting flies to  
381 FS12:12 cycle with feeding restricted to night time did not affect activity/rest pattern (Oishi  
382 et al., 2004). However, in each of the T24 cycles, we found that day 1 of the treatment had a  
383 dramatic effect on phase which persisted for 3-4 cycles in FS10:14 and FS8:16. Furthermore,  
384 T26 and T21 FS cycles could bring about a significant lengthening and shortening of period  
385 while only partially synchronizing the activity/rest rhythm to the external periodicity. These



386 results suggest that while food cannot act as an entraining agent, it affects the clock properties  
387 such as phase and period. This indicates that the clock is perceptive food availability in the  
388 environment and can respond by making small changes to clock properties without changing  
389 the overall pattern of activity/rest.

390 *Drosophila* larvae are known to feed voraciously until they acquire critical weight before  
391 pupation; as adults however, feeding is meager. Nevertheless, when deprived of food, flies  
392 are known to respond by increasing their locomotor activity (Knoppien et al., 2000); (Keene  
393 et al., 2010) which has been attributed to foraging behaviour in flies (Yang et al., 2015).  
394 Indeed, in all our experiments we observed SIH in both male and female flies. Since  
395 hyperactivity is a direct and immediate response to starvation, it can be considered as  
396 masking. In FS10:14 and FS8:16 regimes we found that SIH decreased over subsequent  
397 cycles. This masking to lack of food is also reflected in the phase changes observed during  
398 FS cycles. We observed that day-to-day phase changes indeed mirror the activity levels that  
399 were also higher than baseline during the first few cycles and tapered to baseline in the  
400 subsequent cycles. Since acrophase is a phase marker that depicts the radial centre of mass of  
401 activity, SIH may influence the day-to-day phases. This influence of cyclic starvation  
402 disappeared after release into constant conditions with *ad lib* food. This was evident from the  
403 fact that the  $\Delta$  phase values were found to be significantly different from zero in all three T24  
404 FS paradigms tested. Therefore, while food availability is unable to entrain the activity/rest  
405 rhythm, masking to starvation may bring about some changes which indirectly affects the  
406 phase of the clock. This is of significance to organisms that often encounter unpredictability  
407 in food availability in their environment. Masking to changes in food availability becomes  
408 more relevant for female flies who in addition to their own survival, also need food patches  
409 for laying eggs. In fact, when female flies were subjected to FS8:16 regime, most flies show  
410 excessive activity throughout the period of starvation (Supp fig 4) in contrast with male flies

411 that displayed reduction in SIH midway into the treatment (Fig 1C, c'). Average acrophase  
412 during the FS treatment changes in female flies but they immediately revert to a value similar  
413 to pre-FS cycle when shifted to constant conditions. However, despite SIH and its influence  
414 on the acrophases, the activity/ rest rhythm continues to free run during the FS cycles. This  
415 suggests that in females like in males, masking and free-running components together  
416 regulate day-to-day phases.

417 If flies were consistently masking to lack of food by increasing their activity levels, we would  
418 expect a higher synchrony in inter-individual phases. However, we found that the inter-  
419 individual synchrony was lacking both in males as well as female flies irrespective of short or  
420 prolonged hyperactivity response. Moreover, this increased activity was not only variable  
421 across days but also variable across individuals. Therefore, unlike typically masked  
422 responses which consists of consistent all-or-none responses, in our paradigm we observed a  
423 graded response to lack of food. This graded response may in part explain why inter-  
424 individual synchrony is absent despite occurrence of masking.

425 Food availability has been shown to affect activity patterns of many insects. Solitary bee  
426 species such as carpenter bees and orchid bees show foraging behaviour which is partially  
427 regulated by circadian clocks (Bloch et al., 2017). Social honey bees *Apis mellifera* entrain to  
428 food availability in their environment (Frisch and Aschoff, 1987) underlining the importance  
429 of circadian clocks in anticipating availability of food. Blood mealtimes are carefully phased  
430 to host availability in various hematophagous species such as bed bugs, kissing bugs and  
431 mosquitoes. For example, bed bug *Cimex lectularius* are active during dawn, a time when the  
432 humidity is relatively high and hosts are resting (Barrozo et al., 2004). Similarly, kissing bug  
433 *Triatoma infestans*, the vector for Chagas disease shows peak locomotion, feeding, and  
434 carbon-dioxide sensitivity in the early night presumably when hosts are inactive (Barrozo et  
435 al., 2004). Flight activity and feeding/biting patterns in mosquitoes are influenced by many

436 environmental factors apart from the internal clock mechanisms (Barrozo et al., 2004).  
437 However, these feeding patterns have been shown to change in nocturnal *Anopheles spp* from  
438 peak feeding occurring during the nighttime to a relatively earlier phase due to change in host  
439 availability as a result of interventions to prevent spread of malaria in some African countries  
440 (Reddy et al., 2011); (Gatton et al., 2013);(Sougoufara et al., 2017). Such flexible patterns  
441 are also observed in *Culex pipiens* when blood meals were restricted to a daytime window  
442 (Fritz et al., 2014). Ground beetle *Feronia madida*, have also been previously shown to  
443 change their nocturnal activity patterns to diurnal activity patterns under starved conditions  
444 (Williams, 1959); (Beck, 1980). Cockroach *Periplaneta americana* that have nocturnal  
445 activity and feeding show masking response to food availability when food is restricted to  
446 daytime, however, the activity is still higher during the night suggesting little change to  
447 overall activity/rest pattern (Beck, 1980). Therefore, non-alignment of food availability to an  
448 active state of the organism can influence the activity patterns and possibly the underlying  
449 clock. Additionally, mice gauge the food reward to predation risk ratio and accordingly  
450 change their temporal niche depending upon the environmental conditions (van der Vinne et  
451 al., 2019). Thus, activity patterns may change from nocturnal to diurnal in response to  
452 change in food availability and predation pressure. We sought to ask if similar pressure in  
453 the form of restricted food access to a certain time of day can bring about a change in  
454 activity/rest of *Drosophila melanogaster*. We found that while the clock does not entrain to  
455 the food availability cycle it perceives these changes in food availability and accommodates  
456 them by adjusting its clock properties. This means that even though the activity may free run  
457 during the FS cycles, it free runs with a different phase. Different extent of clock responses  
458 to food could be because of difference in amount of food required for sustenance and/or  
459 difference in natural history and ecology of these species.

460 In mammals, anticipatory response to food availability suggests that circadian clocks are  
461 directly responsive to food availability. While the identity of such a clock (FEO) remains  
462 elusive to date, it is clear that FEO controls food anticipatory activity (Mistlberger, 1994);  
463 (Stephan, 2002). In *Drosophila*, response to restricted food access is masked, which suggests  
464 a homeostatic control. Our study demonstrates that this masked response in the form of SIH,  
465 indirectly affects the activity-controlling central clock. The physiological basis for this  
466 interaction between the homeostatic components and clock components nevertheless has not  
467 been studied so far. Future studies directed at understanding this interaction between clocks  
468 and a food homeostat will help to understand how food availability in the environment can  
469 shape the activity/rest patterns in animals.

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477 **Data availability statement: The data that support the findings of this study are available from**  
478 **the corresponding author upon reasonable request.**

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491 **References**

492 1. Barrozo RB, Schilman PE, Minoli SA, and Lazzari CR (2004) Daily rhythms in  
493 disease-vector insects. *Biol Rhythm Res* 35: 79-92.

494 2. Beck SD (1980) *Insect Photoperiodism*, Second edn (Academic Press, INC).

495 3. Bloch G, Bar-Shai N, Cytter Y, and Green R (2017) Time is honey: circadian clocks  
496 of bees and flowers and how their interactions may influence ecological communities.  
497 *Philos Trans R Soc Lond B Biol Sci* 372.

498 4. Carneiro BT, and Araujo JF (2012) Food entrainment: major and recent findings.  
499 *Front Behav Neurosci* 6: 83.

500 5. Chatterjee A, Tanoue S, Houli JH, and Hardin PE (2010) Regulation of gustatory  
501 physiology and appetitive behavior by the *Drosophila* circadian clock. *Curr Biol* 20:  
502 300-309.

503 6. Connolly KJ (1966) Locomotor activity in *Drosophila* as a function of food  
504 deprivation. *Nature* 209: 224.

- 505 7. Damiola F, Le Minh N, Preitner N, Kornmann B, Fleury-Olela F, and Schibler U  
506 (2000) Restricted feeding uncouples circadian oscillators in peripheral tissues from  
507 the central pacemaker in the suprachiasmatic nucleus. *Genes Dev* 14: 2950-2961.
- 508 8. Diez-Noguera A (2013) Methods for serial analysis of long time series in the study of  
509 biological rhythms. *J Circadian Rhythms* 11: 7.
- 510 9. Dunlap JC, Loros, J. J., & DeCoursey, P. J. (Eds.) (2003) *Chronobiology: Biological*  
511 *timekeeping*. (Sinauer Associates, Inc. Publishers).
- 512 10. Fenske MP, Hewett Hazelton KD, Hempton AK, Shim JS, Yamamoto BM, Riffell  
513 JA, et al. (2015) Circadian clock gene LATE ELONGATED HYPOCOTYL directly  
514 regulates the timing of floral scent emission in *Petunia*. *Proc Natl Acad Sci U S A*  
515 112: 9775-9780.
- 516 11. Fenske MP, Nguyen LP, Horn EK, Riffell JA, and Imaizumi T (2018) Circadian  
517 clocks of both plants and pollinators influence flower seeking behavior of the  
518 pollinator hawkmoth *Manduca sexta*. *Sci Rep* 8: 2842.
- 519 12. Frisch B, and Aschoff J (1987) Circadian-Rhythms in Honeybees - Entrainment by  
520 Feeding Cycles. *Physiol Entomol* 12: 41-49.
- 521 13. Fritz ML, Walker ED, Yunker AJ, and Dworkin I (2014) Daily blood feeding rhythms  
522 of laboratory-reared North American *Culex pipiens*. *J Circadian Rhythms* 12: 1.
- 523 14. Gattton ML, Chitnis N, Churcher T, Donnelly MJ, Ghani AC, Godfray HC, et al.  
524 (2013) The importance of mosquito behavioural adaptations to malaria control in  
525 Africa. *Evolution* 67: 1218-1230.
- 526 15. Hau M, and Gwinner E (1996) Food as a circadian Zeitgeber for house sparrows: the  
527 effect of different food access durations. *J Biol Rhythms* 11: 196-207.

- 528 16. Helfrich-Forster C (1998) Robust circadian rhythmicity of *Drosophila melanogaster*  
529 requires the presence of lateral neurons: a brain-behavioral study of disconnected  
530 mutants. *J Comp Physiol A* 182: 435-453.
- 531 17. Johnson CH, Elliott JA, and Foster R (2003) Entrainment of circadian programs.  
532 *Chronobiol Int* 20: 741-774.
- 533 18. Keene AC, Duboue ER, McDonald DM, Dus M, Suh GS, Waddell S, et al. (2010)  
534 Clock and cycle limit starvation-induced sleep loss in *Drosophila*. *Curr Biol* 20: 1209-  
535 1215.
- 536 19. Knoppien P, van der Pers JNC, and van Delden W (2000) Quantification of  
537 locomotion and the effect of food deprivation on locomotor activity in *Drosophila*. *J*  
538 *Insect Behav* 13: 27-43.
- 539 20. Krishnan B, Dryer SE, and Hardin PE (1999) Circadian rhythms in olfactory  
540 responses of *Drosophila melanogaster*. *Nature* 400: 375-378.
- 541 21. Kronfeld-Schor N, Visser ME, Salis L, and van Gils JA (2017) Chronobiology of  
542 interspecific interactions in a changing world. *Philos Trans R Soc Lond B Biol Sci*  
543 372.
- 544 22. Matile P (2006) Circadian rhythmicity of nectar secretion in *Hoya carnososa*. *Bot Helv*  
545 116: 1-7.
- 546 23. Mistlberger RE (1994) Circadian food-anticipatory activity: formal models and  
547 physiological mechanisms. *Neurosci Biobehav Rev* 18: 171-195.
- 548 24. Mistlberger RE (2011) Neurobiology of food anticipatory circadian rhythms. *Physiol*  
549 *Behav* 104: 535-545.
- 550 25. Moore-Ede M, FM S, and Fuller C (1982) *The Clocks That Time Us: Physiology of*  
551 *the Circadian Timing System*.

- 552 26. Oishi K, Shiota M, Sakamoto K, Kasamatsu M, and Ishida N (2004) Feeding is not a  
553 more potent Zeitgeber than the light-dark cycle in *Drosophila*. *Neuroreport* 15: 739-  
554 743.
- 555 27. Overland L (1960) Endogenous rhythm in opening and odor of flowers of *Cestrum*  
556 *nocturnum*. *Am J Bot* 47: 378-382.
- 557 28. Pendergast JS, and Yamazaki S (2018) The Mysterious Food-Entrainable Oscillator:  
558 Insights from Mutant and Engineered Mouse Models. *J Biol Rhythms* 33: 458-474.
- 559 29. Pittendrigh CS (1960) Circadian rhythms and the circadian organization of living  
560 systems. *Cold Spring Harb Symp Quant Biol* 25: 159-184.
- 561 30. Reddy MR, Overgaard HJ, Abaga S, Reddy VP, Caccone A, Kiszewski AE, et al.  
562 (2011) Outdoor host seeking behaviour of *Anopheles gambiae* mosquitoes following  
563 initiation of malaria vector control on Bioko Island, Equatorial Guinea. *Malar J* 10:  
564 184.
- 565 31. Richter CP (1922) A behavioristic study of the activity of the rat (Baltimore,,  
566 Williams & Wilkins Company).
- 567 32. Romero A, Potter MF, and Haynes KF (2010) Circadian rhythm of spontaneous  
568 locomotor activity in the bed bug, *Cimex lectularius* L. *J Insect Physiol* 56: 1516-  
569 1522.
- 570 33. Schmid B, Helfrich-Forster C, and Yoshii T (2011) A new ImageJ plug-in  
571 "ActogramJ" for chronobiological analyses. *J Biol Rhythms* 26: 464-467.
- 572 34. Sharma VK, Chidambaram R, Subbaraj R, and Chandrashekar MK (2000) Effects  
573 of restricted feeding cycle on the locomotor activity rhythm in the mouse *Mus*  
574 *booduga*. *Physiol Behav* 70: 81-87.



- 575 35. Sougoufara S, Doucoure S, Backe Sembene PM, Harry M, and Sokhna C (2017)  
576 Challenges for malaria vector control in sub-Saharan Africa: Resistance and  
577 behavioral adaptations in Anopheles populations. *J Vector Borne Dis* 54: 4-15.
- 578 36. Stephan FK (2002) The "other" circadian system: Food as a zeitgeber. *J Biol Rhythm*  
579 17: 284-292.
- 580 37. Stephan FK, Swann JM, and Sisk CL (1979) Entrainment of circadian rhythms by  
581 feeding schedules in rats with suprachiasmatic lesions. *Behav Neural Biol* 25: 545-  
582 554.
- 583 38. Tanoue S, Krishnan P, Krishnan B, Dryer SE, and Hardin PE (2004) Circadian clocks  
584 in antennal neurons are necessary and sufficient for olfaction rhythms in *Drosophila*.  
585 *Curr Biol* 14: 638-649.
- 586 39. van der Vinne V, Tachinardi P, Riede SJ, Akkerman J, Scheepe J, Daan S, et al.  
587 (2019) Maximising survival by shifting the daily timing of activity. *Ecol Lett* 22:  
588 2097–2102.
- 589 40. Williams G (1959) Seasonal and Diurnal Activity of Carabidae, with Particular  
590 Reference to *Nebria*, *Notiophilus* and *Feronia*. *J Anim Ecol* 28: 309-330.
- 591 41. Wong R, Piper MD, Wertheim B, and Partridge L (2009) Quantification of food  
592 intake in *Drosophila*. *PLoS One* 4: e6063.
- 593 42. Xu K, Zheng X, and Sehgal A (2008) Regulation of feeding and metabolism by  
594 neuronal and peripheral clocks in *Drosophila*. *Cell Metab* 8: 289-300.
- 595 43. Yang Z, Yu Y, Zhang V, Tian Y, Qi W, and Wang L (2015) Octopamine mediates  
596 starvation-induced hyperactivity in adult *Drosophila*. *Proc Natl Acad Sci U S A* 112:  
597 5219-5224.

598 44. Yon F, Joo Y, Cortes Llorca L, Rothe E, Baldwin IT, and Kim SG (2016) Silencing  
599 *Nicotiana attenuata* LHY and ZTL alters circadian rhythms in flowers. *New Phytol*  
600 209: 1058-1066.

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615 **Figure Legends:**

616 **Figure 1: Flies subjected to FS cycles display a combination of free running and**  
617 **masking behaviour.**

618 Representative double plotted actograms of (**left**) age matched disturbance controls and flies  
619 subjected to FS cycles; (**A**) FS12:12, (**B**) FS10:14, (**C**) FS8:16 and (**right**) average profiles of  
620 activity binned in 15 min intervals (**top**) before and (**bottom**) during FS cycles, (**A**) FS12:12,  
621 (**B**) FS10:14, (**C**) FS8:16. Error bars for activity profiles are  $\pm$  standard error of the mean  
622 (SEM). Yellow shaded region represents daytime and grey shaded region represents night-  
623 time with *ad libitum* food. Pink shaded region represents starvation. In every regime,  
624 disturbance caused due to transfer of flies in fresh tubes results in small bursts in activity,  
625 arrows indicate the startle bouts. In FS 8:16, (c') a small fraction of flies (7/23) shows  
626 gradual reduction in activity (arrowhead) during the starvation window across days of the  
627 treatment; (c'') another fraction of flies (14/23) displays elevated activity levels across all  
628 days of the treatment. *x*-axis indicates external time.

629

630 **Figure 2: Acrophases change across days in flies experiencing FS10:14 & FS8:16**

631 Mean acrophases  $\pm$  SEM of controls (black line) and FS flies (blue line) across days under  
632 LD 12:12, FS treatment and DD *ad lib*, (**A**) FS12:12, (**B**) FS10:14 and (**C**) FS 8:16. Error  
633 bars are standard error of the mean. Experimental flies and their controls show phase change  
634 on day 1 of the regime. Controls revert to previous phase immediately while experimental  
635 flies gradually return to phases similar to controls. Letters denote significant differences ( $p <$   
636  $0.05$ ) between controls and FS flies. \*, \*\*, \*\*\* denote significant differences across days  
637 with  $p < 0.05, 0.01, 0.001$  respectively.

638 **Figure 3: FS cycles are inefficient in exerting phase control and do not result in greater**  
639 **phase synchrony among flies**

640 **(A)** Polar plots depicting average acrophases of individuals for the last 3 days during FS12:12.  
641 black circles indicate phases of control flies and red circles indicate phases of experimental  
642 flies. Zero degrees in each polar plot was set to ZT18 of the previous LD cycles. **(B- Left)**  
643 Scatter plots depicting showing differences in acrophases ( $\Delta$  phase) of individual control  
644 (black) and experimental (red) flies; horizontal line represents the mean  $\Delta$  phase. Horizontal  
645 dotted lines at 0 indicates no change in phase. **(B- Right)** Bar graph depicts mean acrophase  
646  $\pm$  SEM (h) of disturbance controls (black) and experimental (red) flies on the last 3 days.  
647 Degree of phase dispersion, phase change and mean phase in **(C, D)** FS10:14 and **(E, F)**  
648 FS8:16 respectively, all other details same as **(A & B)**. \*,\*\* indicate  $p < 0.05, 0.01$   
649 respectively.

651 **Figure 4: Phase shifted FS cycles do not reveal any difference in inter-individual phase**  
652 **synchrony**

653 **(A)** Polar plots depicting averaged phases for the last 3 days of individual flies during  
654 FS12:12. **(Left)** black circles indicate phases of control flies and **(right)** red circles indicate  
655 the phase of flies subjected to the first FS12:12 (FS1). **(Bottom)** Polar plots depicting  
656 average phases (last 3 days) of **(left)** control and **(right)** experimental individuals during FS2,  
657 which was phase *delayed* by 6 hours compared to FS1. The degree of dispersion of  
658 acrophases in disturbance controls and experimental flies was not significantly different  
659 during FS1 and FS2. **(B)** Polar plots depicting averaged phases for the last 3 days of  
660 individual flies during the first FS12:12 regime (FS1). Polar plots depicting average phases  
661 (last 3 days) of control and experimental individuals during FS2, which was phase *advanced*  
662 by 6 hours compared to FS1. The degree of dispersion of acrophases in disturbance controls

663 and experimental flies was not significantly different during FS1 and FS2. Scatter plots  
664 showing  $\Delta$  phase for individual control (black) and experimental (red) flies when phase on  
665 the first day in DD *ad libitum* was subtracted from averaged phase in the last 3 days of (C)  
666 phase delayed and (D) advanced FS2 cycles \*,\*\* indicate  $p < 0.05, 0.01$  respectively. Other  
667 details as in Figure 3.

668

669 **Figure 5: FS T26 cycles do not entrain the locomotor activity/rest rhythm.**

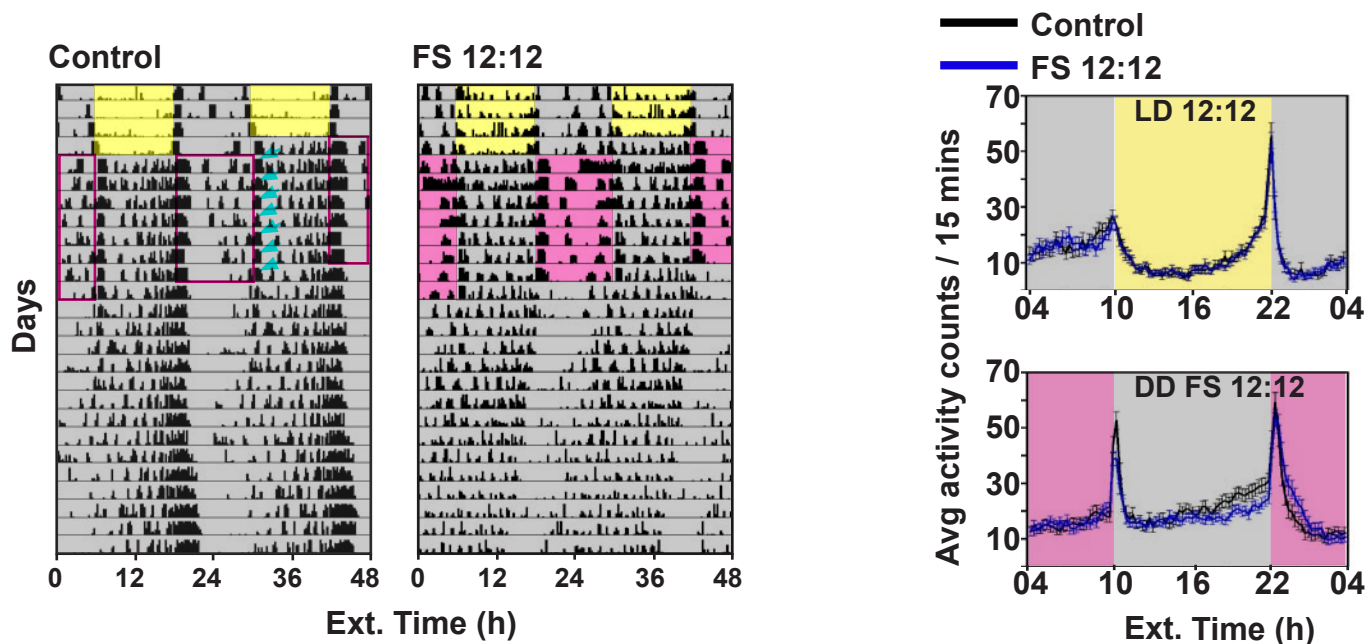
670 (A) Representative actograms of (left) control and (right) experimental flies recorded under  
671 LD12:12 conditions for the first 4 days before being subjected to T26 FS cycles in DD  
672 conditions. Pink shaded region represents 13 hours of starvation on each day. T26 FS cycles  
673 continued for 7 days followed by DD *ad libitum* conditions. Both the control and the  
674 experimental flies show masked responses to the T26 regime along with a free running  
675 component of activity ( $^{24}\tau_1$ ) that seems to follow the phase from the LD12:12 cycle. Dashed  
676 line tracks the startle activity due to food changes (green) and dotted line tracks activity  
677 offset phase after LD 12:12 (red). (B) Proportion of flies showing two different periodicities  
678 (26 h and 24 h), only 26 h or only 24 h periodicity or arrhythmicity during T26 FS are  
679 indicated. A major proportion of both control and experimental flies show dual periodicities.  
680 (C) Distribution of changes in the  $\sim 24$  h periodicity during the T26 regime for controls and  
681 experimental flies. Differences were calculated by subtracting periods after T26 FS ( $^{24}\tau_2$ )  
682 from the periods during T26 FS ( $^{24}\tau_1$ ). T26 FS flies have a longer free-running component  
683 ( $\sim 24$  h) during T26. NS denotes no difference between controls and T26 FS flies. \*\*\*  
684 denotes  $p < 0.001$ .

685

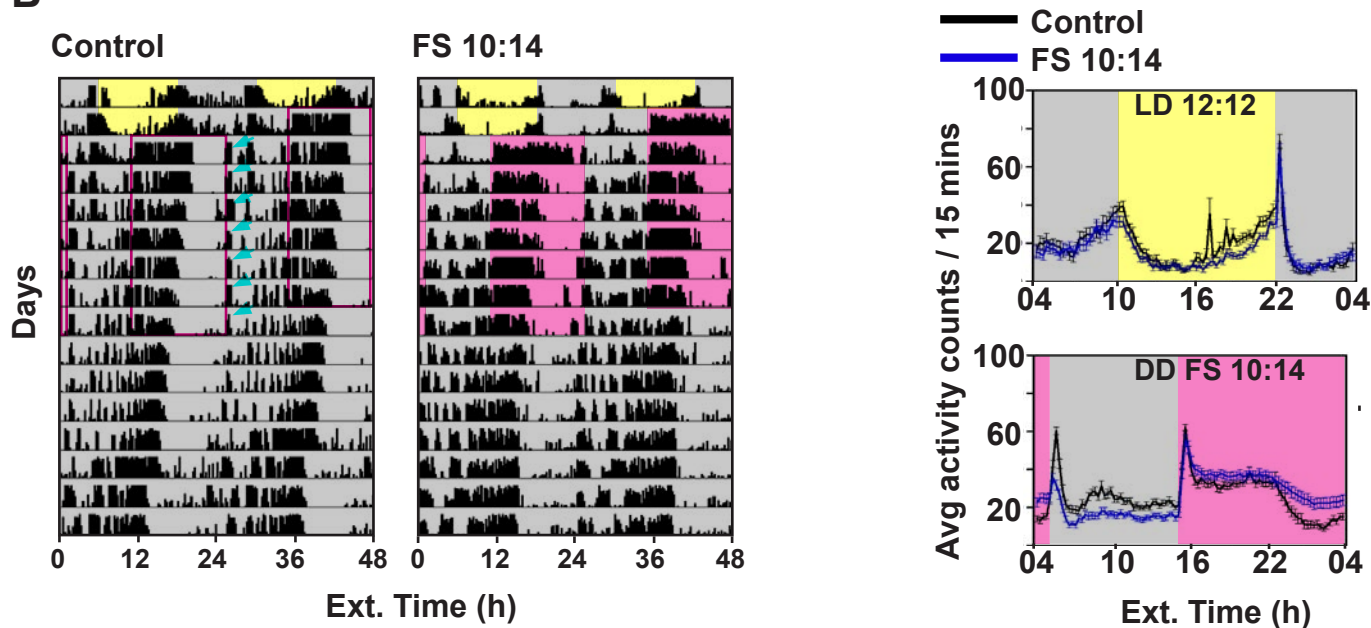
686 **Figure 6: T21 cycles do not entrain the locomotor activity/rest rhythm in *D.***  
687 ***melanogaster***

688 **(A)** Representative actograms of **(left)** control and **(right)** experimental flies recorded in LD  
689 12:12 conditions for the first 4 days before being subjected to T21 FS cycles in DD. Pink  
690 shaded region represents 10.5 hours of starvation on each day. T21 FS cycles continued for 7  
691 days followed by DD *ad libitum* conditions. Both the control and the experimental flies show  
692 masked responses to the T21 regime along with a free running component of activity ( $^{24}\tau_1$ )  
693 that seems to follow the phase from the LD12:12 cycle. **(B)** Proportion of flies showing two  
694 different periodicities (21 h and 24 h), only 21 h or only 24 h periodicity or arrhythmicity  
695 during T21 FS are indicated. A major proportion of both control and experimental flies show  
696 dual periodicities. A higher proportion of T21 FS flies show only 21 h periodicity. **(C)**  
697 Distribution of changes in the  $\sim 24$  h period during the T21 regime for **(left)** controls and  
698 **(right)** experimental flies. Differences were calculated by subtracting periods after T21 FS  
699 ( $^{24}\tau_2$ ) from the periods during T21 FS ( $^{24}\tau_1$ ). T21 FS flies have a shorter free-running ( $\sim 24$  h)  
700 component during T21. \*\*\* denotes  $p < 0.001$ . All other details as in Figure 5.

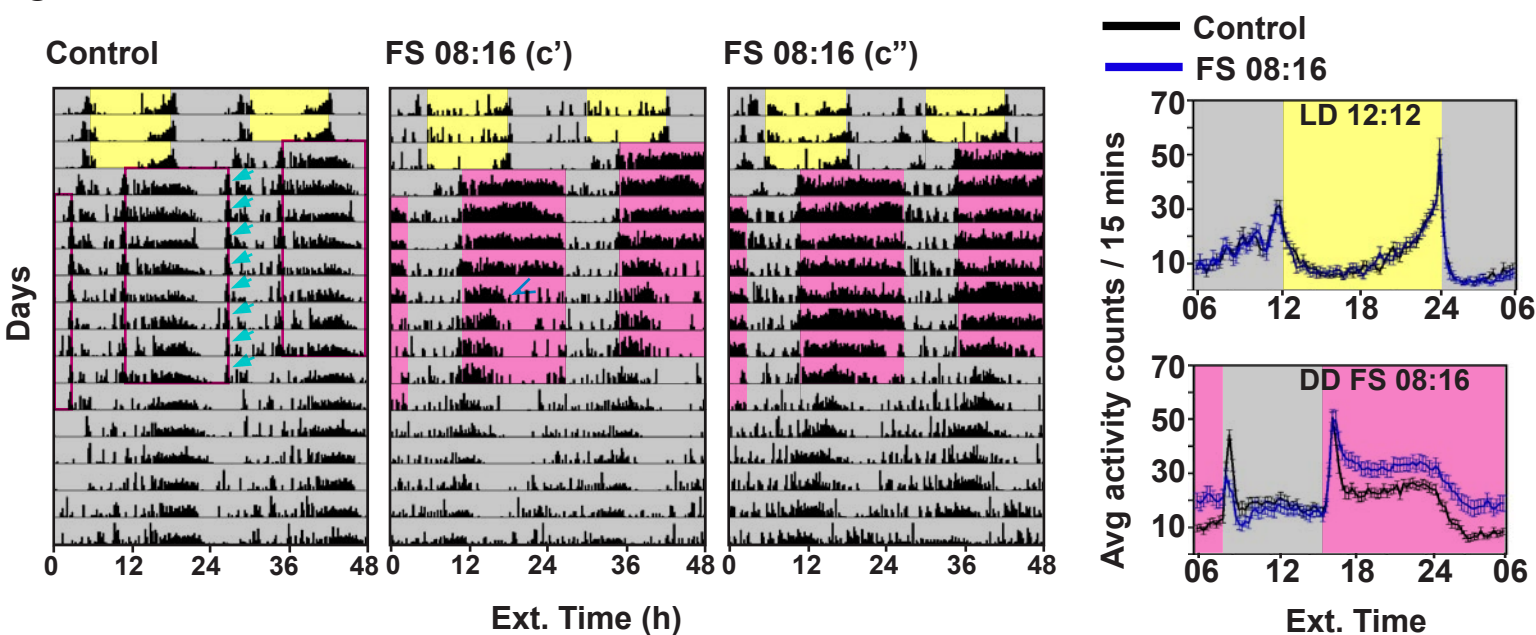
**A**

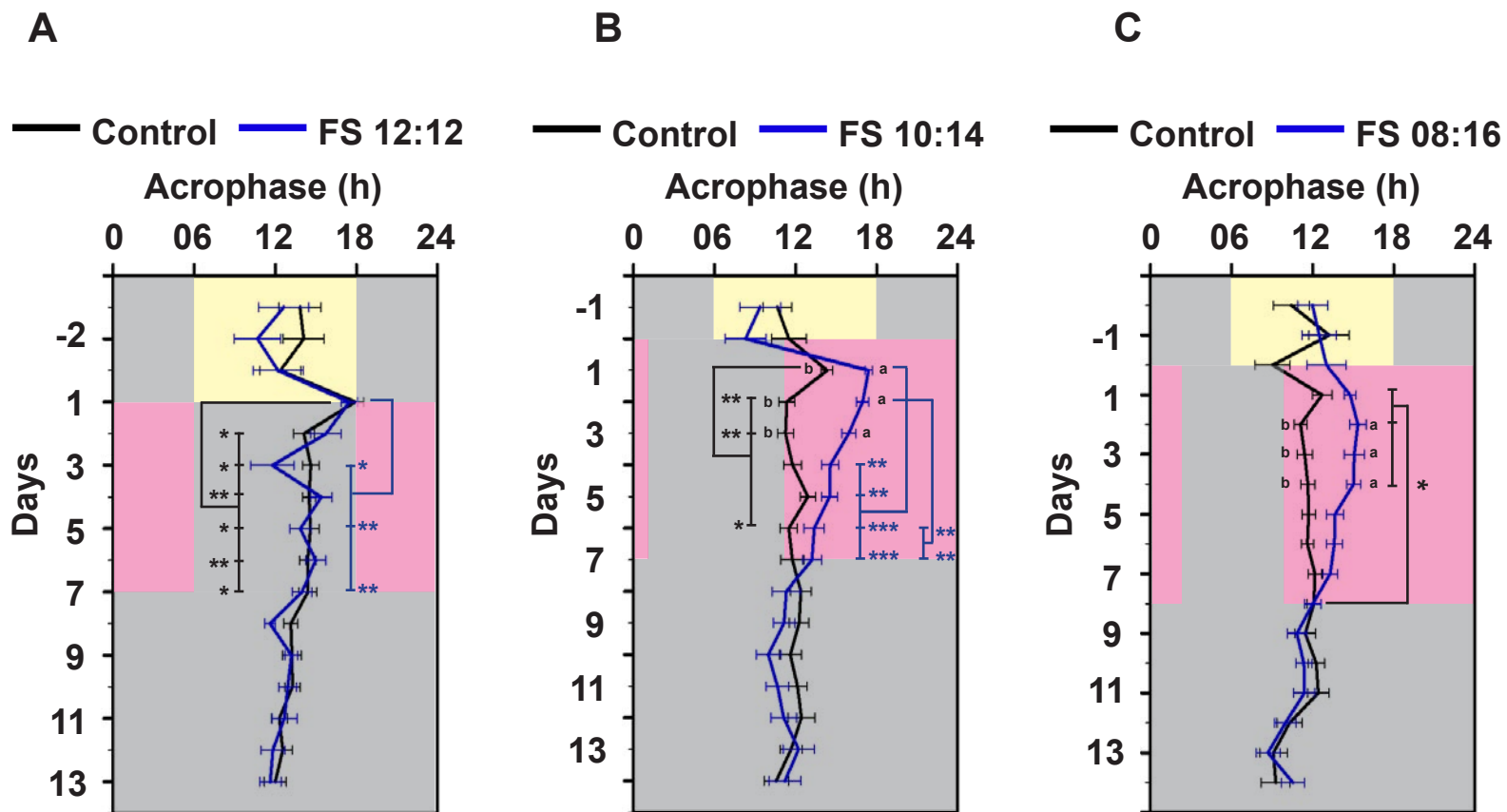


**B**



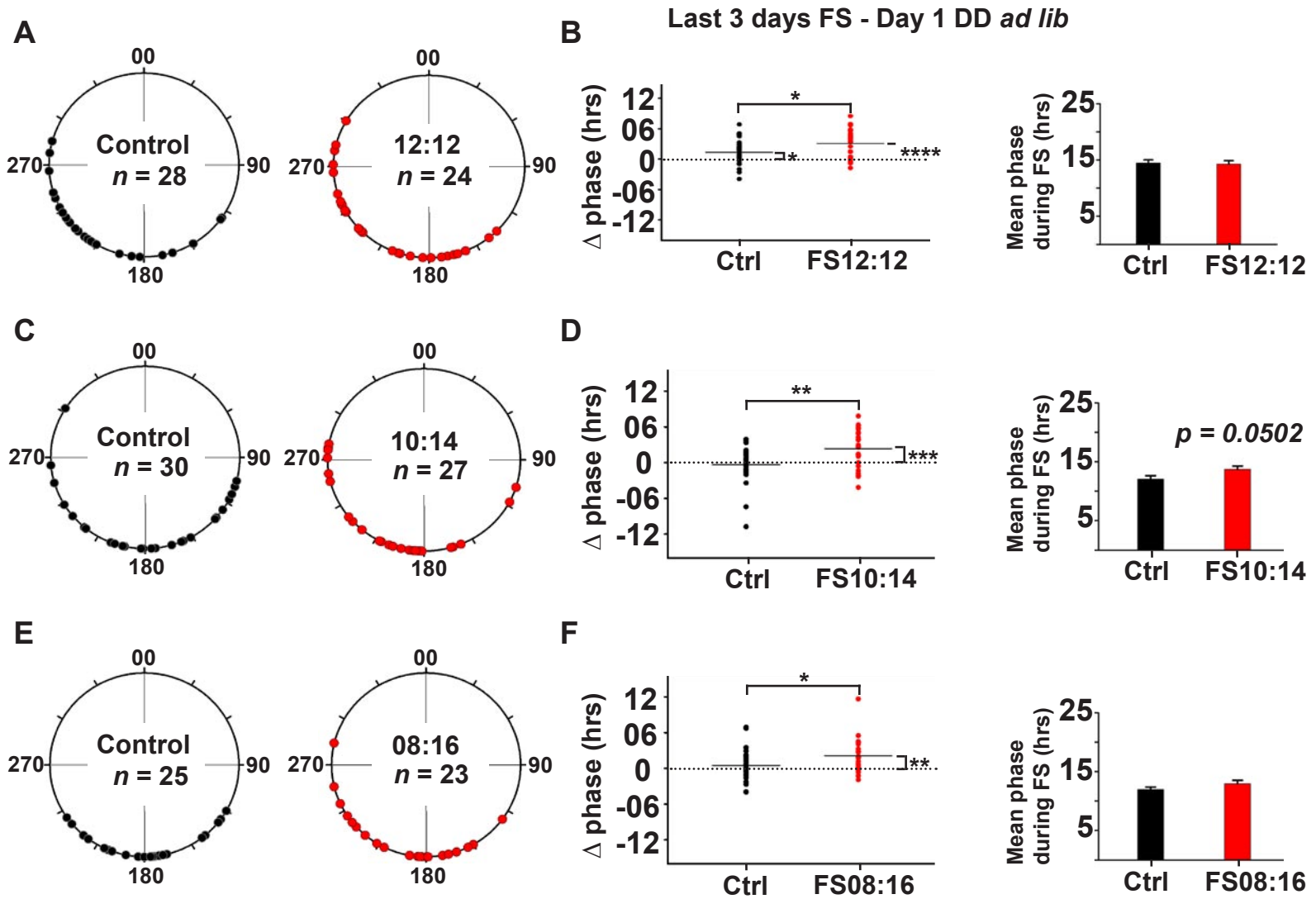
**C**







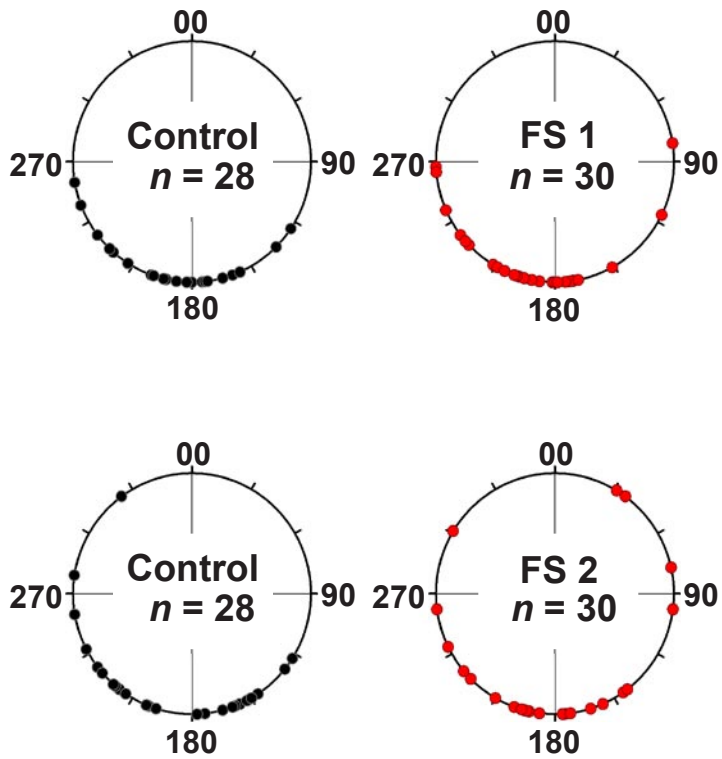
## Males



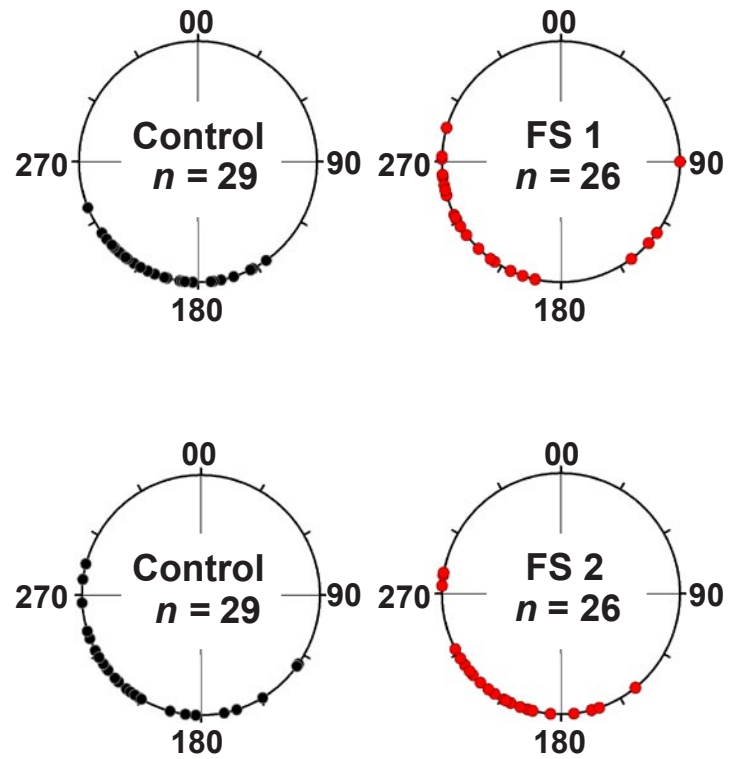
## Phase Delay

## Phase Advance

**A**

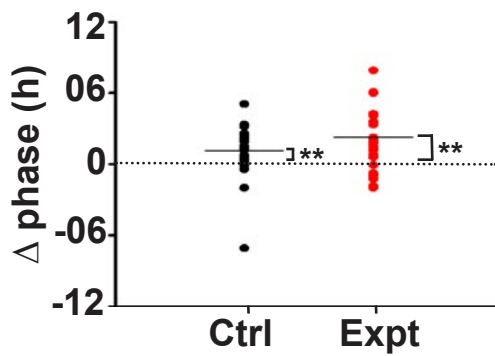


**B**



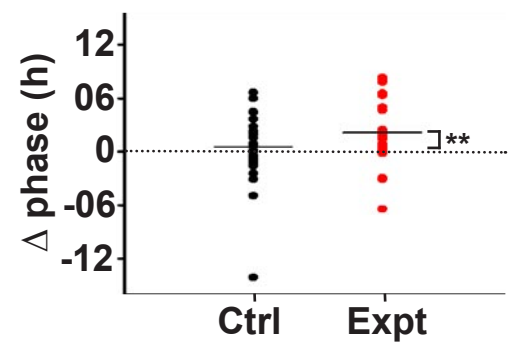
**C**

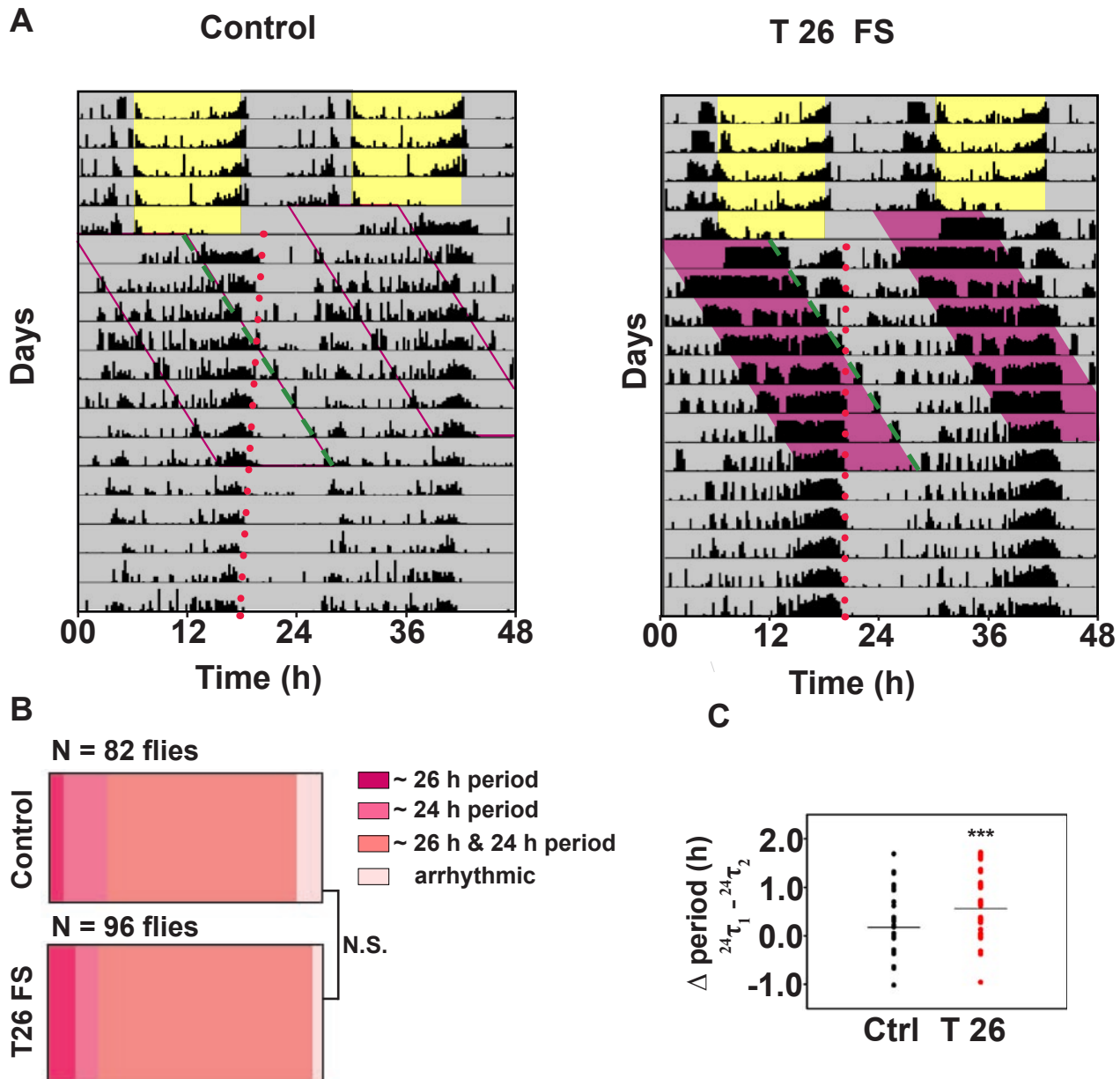
Last 3 days FS2 - Day 1 DD *ad lib*

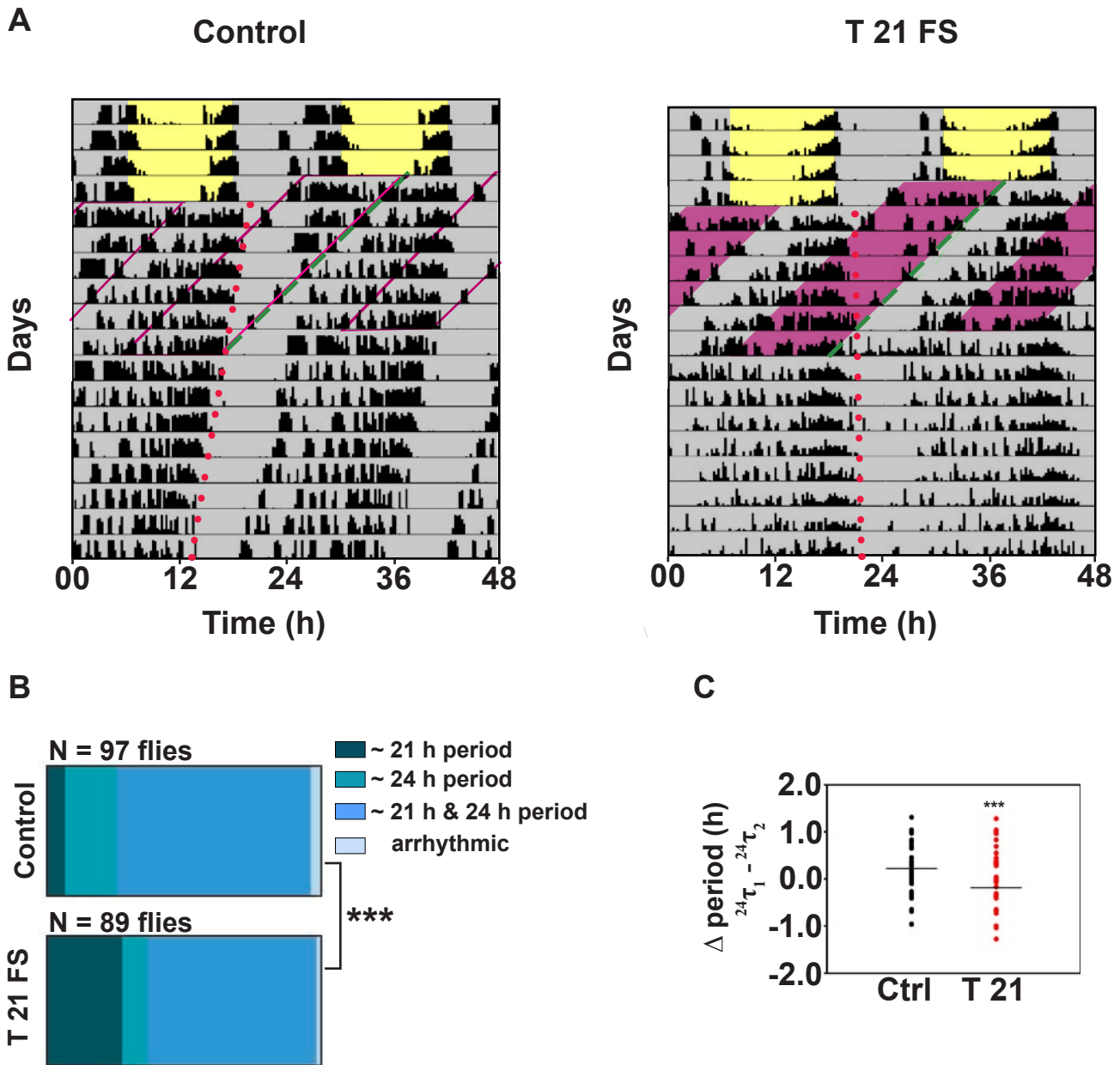


**D**

Last 3 days FS2 - Day 1 DD *ad lib*







## Supplementary online material

### Effects of food availability cycles on phase and period of activity/rest rhythm in

#### *Drosophila melanogaster*

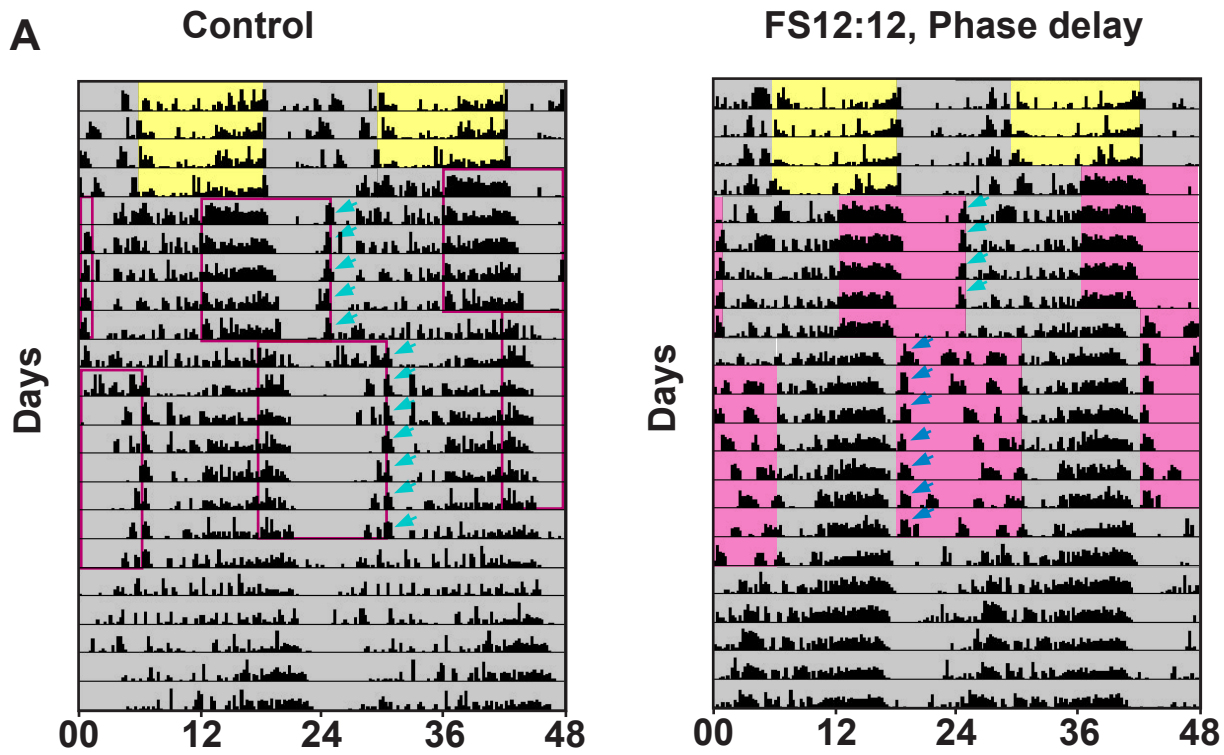
Viveka Jagdish Singh<sup>1</sup>, Sheetal Potdar<sup>1#</sup>, Vasu Sheeba<sup>2\*</sup>

**Affiliation:** Chronobiology and Behavioural Neurogenetics Laboratory, Evolutionary and Integrative Biology Unit<sup>1</sup>, Neuroscience Unit<sup>2</sup>, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore

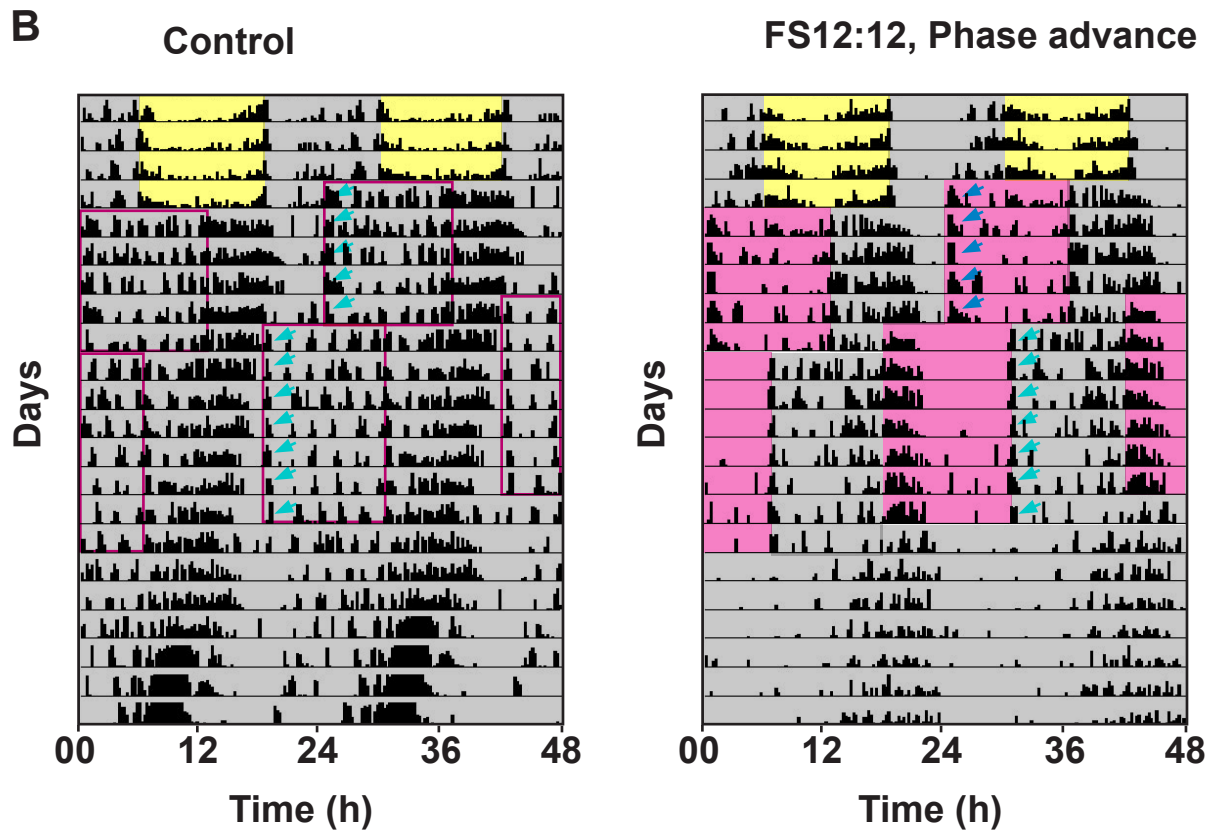
<sup>#</sup>Current affiliation: Molecular Neurobiology Laboratory, Salk Institute for biological studies, La Jolla, CA 92037

**\*Corresponding author:** Sheeba Vasu

## 6 hour phase delayed cycles

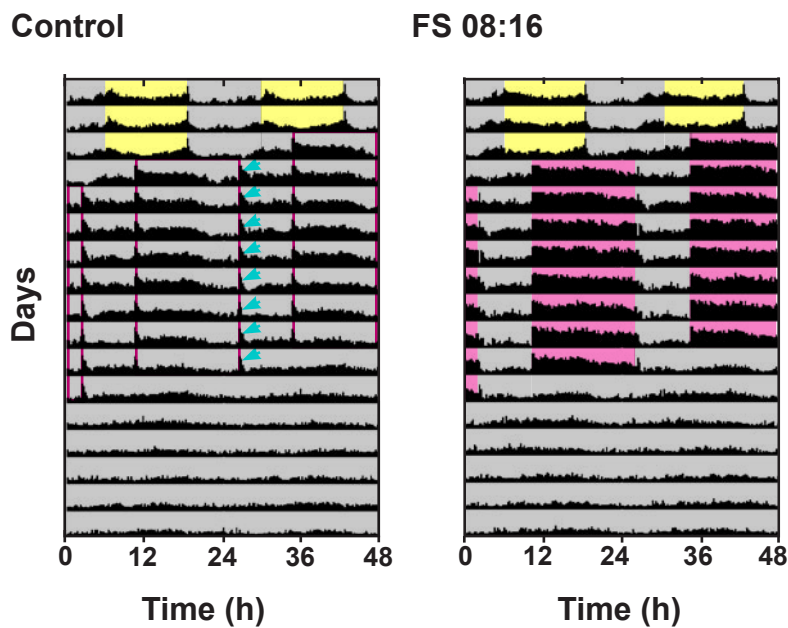


## 6 hour phase advanced cycles

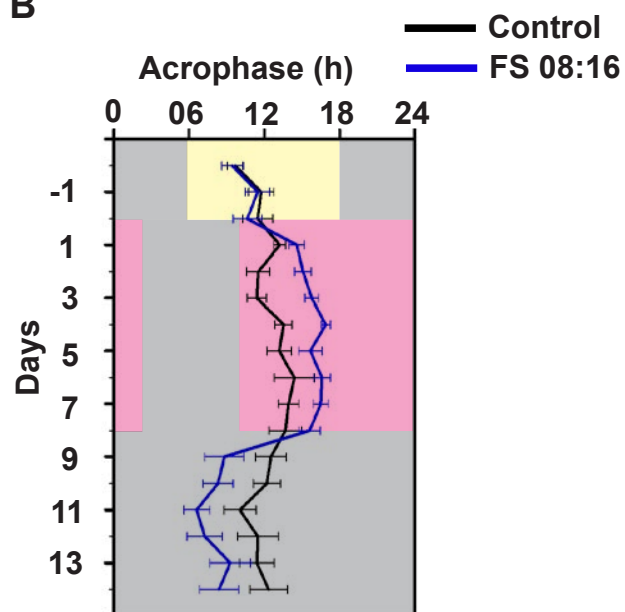


## Females

**A**

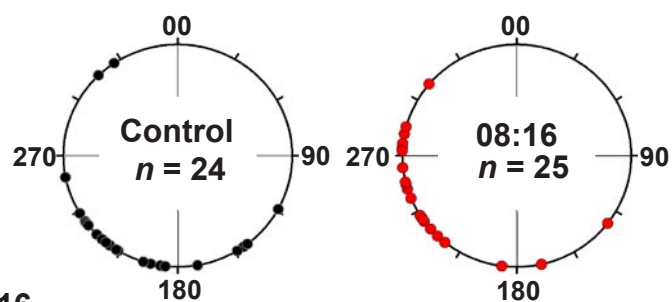
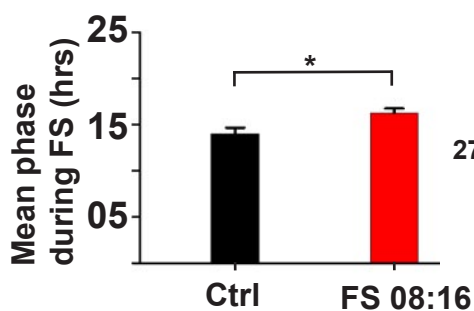
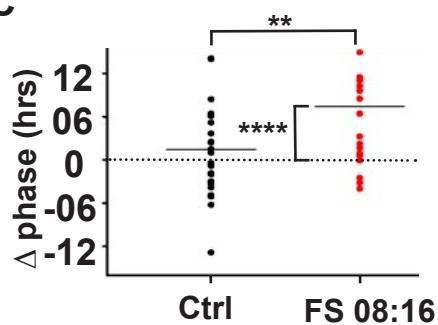


**B**



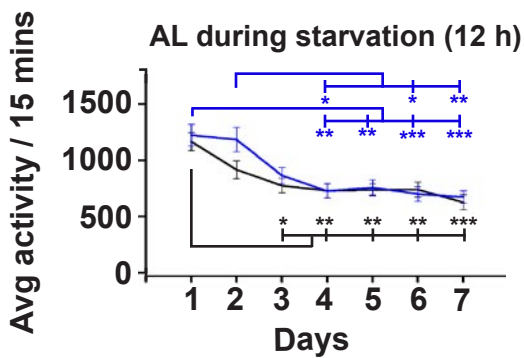
Last 3 days FS - Day 1 DD *ad lib*

**C**



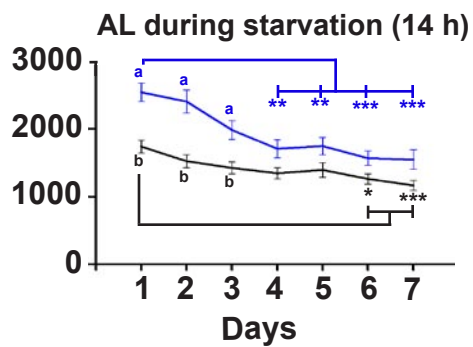
**A**

— FS 12:12    — Ctrl



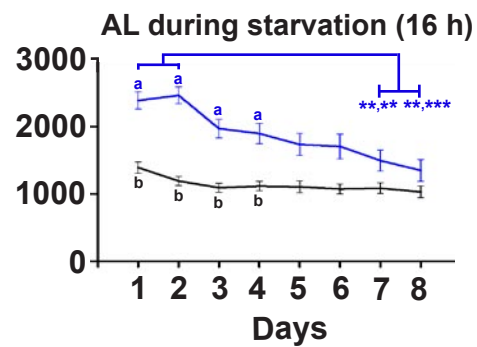
**B**

— FS 10:14    — Ctrl



**C**

— FS 08:16    — Ctrl





1 **Figure legends:**

2 **Supplementary Figure 1: Phase delayed and phase advanced FS cycles do not**  
3 **synchronize the locomotor activity/rest rhythm in *D. melanogaster***

4 **(A)** Representative double plotted actograms of age matched disturbance control flies **(left)**  
5 and flies subjected to two consecutive FS12:12 cycles phase delayed to one another **(right)**.  
6 Flies were housed in LD12:12 and later subjected to FS12:12 (pink shaded region depicts  
7 starvation hours) for 5 cycles. Starvation period **(FS1)** began 6 hours phase delayed with  
8 respect to the LD12:12 such that the LD and the FS regime were not in-sync with each other.  
9 FS1 cycles were further delayed by another 6 hours after the first 5 days, and this continued  
10 for another 7 days **(FS2)**. Flies were subsequently released into constant conditions with *ad*  
11 *libitum* food (DD, 25°C). Arrows indicate the startle bouts. **(B)** Representative double  
12 plotted actograms of age matched disturbance control flies **(left)** and flies subjected to two  
13 consecutive FS12:12 cycles phase advanced to one another **(right)**. Flies were subjected to  
14 FS12:12 for 5 cycles in DD. **FS1** was 6 hours phase advanced compared to the initial  
15 LD12:12 regime. FS1 was followed by **FS2** which was further advanced by 6 hours  
16 compared to FS1. All other details are same as **(A)**. Experimental flies fail to synchronise to  
17 FS1 and FS2 in both phase delay and phase advance experiments and continue to display a  
18 phase from the previous LD cycle.

19

20 **Supplementary Figure 2: Female flies do not get entrained to FS8:16 cycles**

21 **(A)** Double plotted batch actograms of **(left)** age matched disturbance control flies and  
22 **(right)** female flies subjected to FS8:16 regime. Flies show a prolonged activity bout on all  
23 cycles of the treatment. **(B)** Mean acrophases  $\pm$  SEM of controls (black line) and FS flies

24 (blue line) across days of LD 12:12, FS8:16 treatment and DD *ad lib*. Experimental flies  
25 show phase change during the regime. Phases revert to previous values immediately after  
26 DD *ad lib*. **(C) (Left)** scatter plots showing  $\Delta$  phases of individual control (black) and  
27 experimental (red) flies, \*, \*\* indicate  $p < 0.05$ ,  $0.01$  respectively. **(Centre)** Bar graph  
28 depicts mean acrophase (h)  $\pm$  SEM during FS8:16 of disturbance controls (black) and  
29 experimental (red) flies in the last 3 days. **(Right)** polar plots depicting average acrophases  
30 for the last 3 days of individual flies during FS 8:16. **(left)** black circles indicate phases of  
31 control flies and **(right)** red circles indicate the phase of experimental female flies that were  
32 subjected to FS8:16 regime. All other details as in Figure 1 & 3.

33

34 **Supplementary Figure 3: FS12:12, 10:14, 08:16 cycles do not result in sustained**  
35 **starvation-induced hyperactivity throughout the period of FS regime**

36 Activity levels during starvation period of each day  $\pm$  SEM during **(A)** FS12:12, **(B)** FS10:14,  
37 **(C)** FS8:16. Starvation induced hyper-activity in the experimental flies progressively reduces  
38 during the latter half of the 10:14, 8:16 FS cycles. Letters denote significant differences ( $p$   
39  $< 0.05$ ) between controls and FS flies. \*, \*\*, \*\*\* denote  $p < 0.05$ ,  $0.01$ ,  $0.001$  respectively.

40

**Table:1 Period and amplitude values of control and experimental flies during and after FS 12:12, FS 10:14 and FS 8:16 cycles**

MALES								
Regime	Period		Power		n		% Rhythmicity	
	Control	Expt.	Control	Expt.	Control	Expt.	Control	Expt.
During FS 12:12 1	23.8 ± 0.05	23.7 ± 0.05	167.07 ± 6.9	166.48 ± 5.7	29	32	100	100
During FS 12:12 2 (phase delayed)	24.02 ± 0.04	23.99 ± 0.02	203.7 ± 8.4	190.04 ± 7.2	29	32	100	100
DD <i>ad lib</i> after FS 12:12	23.83 ± 0.07	23.72 ± 0.07	170.8 ± 12.6	156.5 ± 12.2	28	30	96.43	96.67

Regime	Period		Power		n		% Rhythmicity	
	Control	Expt.	Control	Expt.	Control	Expt.	Control	Expt.
During FS 10:14	23.91 ± 0.04	23.84 ± 0.06	205.3 ± 6.6	229.7 ± 8.8	30	28	100	100
DD <i>ad lib</i> after FS 10:14	23.67 ± 0.04	23.62 ± 0.08	210.38 ± 14.4	237.94 ± 16.1	30	27	87.09	88.89

Regime	Period		Power		n		% Rhythmicity	
	Control	Expt.	Control	Expt.	Control	Expt.	Control	Expt.
During FS 08:16	23.97 ± 0.02	23.95 ± 0.02	249.9 ± 9.43	243.85 ± 10.1	32	23	100	100
DD <i>ad lib</i> after FS 08:16	23.76 ± 0.09	23.70 ± 0.08	120.11 ± 6.6	122.31 ± 6.6	32	23	87.5	95.65

FEMALES								
Regime	Period		Power		n		% Rhythmicity	
	Control	Expt.	Control	Expt.	Control	Expt.	Control	Expt.
During FS 12:12 1	23.8 ± 0.07	23.99 ± 0.14	131.04 ± 5.28	126.49 ± 4.9	28	29	85.71	93.1
During FS 12:12 2 (phase delayed)	23.89 ± 0.26	24.46 ± 0.41	155.65 ± 10.52	140.87 ± 7.19	26	27	73.08	81.5
DD <i>ad lib</i> after FS 12:12	23.96 ± 0.11	23.6 ± 0.16	125.75 ± 11.3	103.5 ± 8.52	23	25	52.2	26.09*

Regime	Period		Power		n		% Rhythmicity	
	Control	Expt.	Control	Expt.	Control	Expt.	Control	Expt.
During FS 10:14	24 ± 0.02	23.92 ± 0.05	164.8 ± 7.3	179.2 ± 9.8	25	25	100	100
DD <i>ad lib</i> after FS 10:14	23.6 ± 0.15	23.4 ± 0.54	169.18 ± 13.23	152.8 ± 10.93	20	25	60	40

Regime	Period		Power		n		% Rhythmicity	
	Control	Expt.	Control	Expt.	Control	Expt.	Control	Expt.
During FS 08:16	24.15 ± 0.1	24.3 ± 0.24	160.5 ± 9.07	215.77 ± 13.1	24	25	95.83	96
DD <i>ad lib</i> after FS 08:16	23.75 ± 0.21	23.36 ± 0.18	98.4 ± 6.6	98.65 ± 9.53	20	16	40	37.5

**Table:2 Number of flies showing two periodicities when subjected to FS T26**

	n	No. of flies with 2 periodicities ~24h & ~26	Flies exhibiting only ~ 26 h periodicity	Flies exhibiting only ~ 24 h periodicity	Flies exhibiting >2 periodicities	Arrhythmic flies	Average period (~24 h)	Average period (~26 h)
Control	94	57	5	13	7	7	23.94 ± 0.06	25.96 ± 0.02
T26	120	75	10	8	14	3	24.27 ± 0.14	25.98 ± 0.004

**Table:3 Number of flies showing two periodicities when subjected to FS T21**

	n	No. of flies with 2 periodicities ~24h & ~21h	Flies exhibiting only ~ 21 h periodicity	Flies exhibiting only ~ 24 h periodicity	Flies exhibiting >2 periodicities	Arrhythmic flies	Average period (~ 24 h)	Average period (~ 21 h)
Controls	108	69	7	18	11	3	24.03 ± 0.05	20.97 ± 0.009
T21	95	55	25	8	6	1	23.82 ± 0.03	21.01 ± 0.02