Regulation of starvation-mediated hyperactivity and triglyceride metabolism by Short neuropeptide F and Insulin Like Peptide in *Drosophila*

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Running title: sNPF and DILP2 govern starvation-mediated hyperactivity and triglyceride metabolism in fruit flies.

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Abstract:

Circadian clock regulates various behavioral, metabolic and physiological processes to occur at the most suitable time of the day. The most apparent behavioral outputs of the clock are activity-rest rhythm and feeding. While clock regulates these two behaviors through interconnected neuronal circuits, the precise pathway through which the clock coordinates metabolism in accordance with the behavioral rhythms largely remains to be elucidated. This study was aimed to elucidate the role of two circadian relevant metabolic neuropeptides, short neuropeptide F (sNPF) and *Drosophila* Insulin Like Peptide 2 (DILP2) in triglyceride metabolism, starvation-mediated hyperactivity, and food intake in *Drosophila*. The results showed that *snpf* transcripts exhibit significant rhythmicity under 12:12 hour light-dark cycles (LD) and constant darkness (DD). Knockdown of sNPF in sNPF producing neurons enhanced the starvation-mediated hyperactivity in flies compared to the control. Further studies showed that sNPF suppresses starvation-induced hyperactivity partly through the sNPF receptors (sNPFR) expressed in insulin producing cells (IPC). Knockdown of *Drosophila* insulin like peptide 2 (*dilp2*) in IPCs and reduced expression of the insulin receptors (InR) in the fat body altered the starvation-mediated hyperactivity, lipid storage, and increased the food intake after starvation. These results suggest a role for sNPF in modulating locomotor activity in accordance with the nutrient availability and a novel role for circadian output molecule DILP2 in regulating triglyceride metabolism and starvation-mediated behavioral changes in *Drosophila*.

Keywords: circadian, short neuropeptide F, *Drosophila* Insulin Like Peptide 2, starvation, triglyceride metabolism

Introduction

The circadian clock drives daily rhythms in a wide array of physiological and behavioral processes by scheduling it at the appropriate time of the day. This inherent timekeeping system coordinates the phase of two fundamental behavioral rhythms such as feeding and sleep/wake cycles and it is believed to aid the organism to adapt with the cyclic external environmental changes. The growing body of evidence indicates that the circadian clock not only mediates optimal phasing of behavioral rhythmicity during 24 hour of a day but also has intimate ties with metabolism [1]. While the clock orchestrates numerous metabolic pathways, the nutrient availability and metabolic status can in turn feed back to impinge on the functioning of the circadian clock [2], [3], [4], [5]. A large body of evidence accumulated over the past two decades remarkably improved our understanding of the vital role of the clock in metabolism, feeding behavior, and sleep wake cycle [6]. However, little is known about the precise underlying pathways by which the circadian clock aids the organism to attain temporal harmonics between behavior and metabolism.

The association between the circadian clock and metabolism was further substantiated by the findings that the circadian timing system drives rhythmicity of various peptides involved in metabolism that also play key role in the regulation of sleep wake cycles [7]. For instance, peptides involved in metabolism such as leptin, ghrelin and cholecystokinin display circadian rhythmicity in expression [8], [9], [10], [11]. Another appetite stimulating hormone orexin promotes wakefulness while stimulated by ghrelin and enhances sleep when inhibited by leptin [12] and orexigenic neuron activation is believed to be under circadian control [13]. In addition

64 to regulating the metabolic endocrine rhythms through the anatomical connections that interlink
65 with the centers of sleep wake cycles, the cross talk between the master clock in the brain and the
66 peripheral clocks located in metabolically active tissues is believed to harmonize the metabolic
67 homeostasis [14]. In *Drosophila*, the peripheral clocks in the fat body mediates optimal phase of
68 feeding rhythm, regulates the starvation sensitivity, triglyceride and glycogen levels, drive
69 rhythmicity in the expression of genes involved in metabolism and detoxification to achieve
70 energy homeostasis [5] [15] [16]. Although it was reported that central clock neurons oppose the
71 effect of the fat body peripheral clock on energy storage [15], the potential mediators by which
72 the master clock communicates the time of the day information to the fat body to achieve energy
73 homeostasis remains to be largely achieved.

74 A recent study showed that central clock neurons regulate rhythmic gene expression in
75 the fat body through neuropeptide F (NPF) in *Drosophila* [17]. Moreover, pars intercerebralis
76 (PI) has been identified as an important clock output region that regulates activity rest rhythms
77 [18] and the dorsal clock neurons (DN1) are physically and functionally connected to the insulin
78 producing cells (IPCs) of the PI. *Drosophila* insulin like peptide 2 (DILP2) produced in IPCs is
79 an identified clock output-signaling molecule that drives rhythmicity in the expression of lipase
80 transcript in the fat body [19]. Furthermore, DILPs and insulin signaling play a pivotal role in
81 feeding behavior [20], [21], [22]. Feeding behavior displays a circadian rhythm and its phase is
82 most likely regulated by the peripheral clock present in the fat body [16]. Thus, it is conceivable
83 that the cross talk between the master clock and the peripheral clock in the fat body may
84 optimize the phase of both feeding and lipid metabolism to occur at a specific part of the day.
85 However, the precise interdependent and independent pathways/networks by which the circadian
86 clock regulates feeding behavior and lipid metabolism remain to be elucidated. It is likely that

87 the circadian regulation of metabolism and feeding behavior are intertwined to some degree and
88 the insulin signaling could be one of the primary clock output peptidergic signals involved in this
89 cross modulation.

90 Previous studies showed that short neuropeptide F (sNPF) and sNPF receptor (sNPFR)
91 signaling in IPCs regulate the expression of *dilps* [23]. In addition, sNPF regulates circadian
92 rhythm, sleep and feeding behavior in *Drosophila* [24], [25]. Feeding and sleep wake cycles are
93 interconnected behaviors and the optimum balance between them is disrupted under nutrient
94 deprivation. Starvation suppresses sleep by promoting hyperactivity in *Drosophila* and this
95 behavioral change in response to nutrient unavailability is governed by diverse neuropeptides
96 such as allatostatin, octopamine, adipokinetic hormone, glucagon and insulin [26], [27], [28],
97 [29]. While significant progress has been made in understanding the circadian and neuronal
98 basis of starvation-mediated sleep changes in *Drosophila* [30], it remains unclear whether the
99 circadian clock conveys the time of the day information to coherently coordinate this starvation-
100 mediated behavioral change with metabolism through interlinked neuronal pathways or via
101 independent neural circuitry. In the present study, we aimed to elucidate the role of circadian
102 clock in regulating the metabolic neuropeptides and the specific role of neuropeptides sNPF and
103 DILP2 in triglyceride metabolism, starvation-mediated hyperactivity, and feeding behavior in
104 *Drosophila*. The results showed that the circadian clock regulates the transcription of *snpf* gene
105 and sNPF suppresses starvation-mediated hyperactivity in *Drosophila*. Further studies
106 deciphered the vital role of DILP2 in IPCs and Insulin receptors (*InR*) expressed in the fat body
107 in mediating the starvation-mediated hyperactivity, triglyceride metabolism and hunger induced
108 food intake in *Drosophila*.

109 Materials and Methods

Fly stocks and maintenance

Following fly lines were used in this experiment: w^{1118} (BDSC #5905), *dilp2-Gal4/CyOGFP* (Stephen Cohen Laboratory), *tim-Gal4* (Sheeba Vasu Laboratory), *CG-Gal4* (BDSC #7011), *snpf-Gal4*, *dilp2-Gal4 GFP*, UAS-$snpf^{RNAi}$ (Kweon Yu Laboratory), *UAS-dilp2* ^{RNAi} (VDRC #102158), *UAS-InR* ^{DN} (II) (BDSC #8252) were obtained from Bloomington Drosophila Stock Center (Bloomington, IN). All fly stocks were maintained in the *Drosophila* growth chamber (MIR-154, Panasonic, Japan) at 25º C temperature, 75 ± 5% humidity and 12:12 hour (h) light-dark (LD) cycle where lights came on at Zeitgeber Time 00 (ZT 00) and went off at ZT12.

Measurement of mRNA levels

To assess the mRNA transcription profiles of *snpf*, three replicates were used for each time point. Each replicate contained 30 heads from w^{1118} flies. Total RNA was extracted using QIAGEN RNAeasy Plus Mini kit; DNAase digestion was performed using QIAGEN RNase–free DNase. cDNA was synthesized using SuperScript™ III First-Strand Synthesis System (Invitrogen, Cat. No. 18080051) and real-time PCR was performed using Bio-Rad CFX96TM with the cDNA template, Power SYBR® Green PCR Master Mix (Cat. # 4368702) from ThermoFisher Scientific to check temporal transcription profile of *snpf*. mRNA levels were measured under LD cycle and constant darkness (DD). The molecular oscillation of mRNA levels was determined by normalizing the mRNA level of the gene of interest with the mRNA level of *rp49* at each time point. Knockdown efficiency of *snpf-Gal4 > UAS* $snpf^{RNAi}$ (*snpf-Gal4 >*$snpf^{RNAi}$) and *dilp2-Gal4 > dilp2*^{RNAi} was validated by qPCR. Primers used for qPCR are listed in Table 1.

Table 1. PCR primers used for quantitative RT-PCR.

133

Primers	Forward	Reverse
dilp2	5'- GGCCAGCTCCACAGTGAAGT -3'	5'- TCGCTGTCGGCACCGGGCAT -3'
Snpf	5'- CCCGAAAACTTTTAGACTCA-3'	5'- TTTTCAAACATTTCCATCG -3′
rp49	5'- GCTAAGCTGTCGCACAAA-3'	5'- TCCGGTGGGCAGCATGTG-3'

134

135 # Activity/rest assay

136 For activity/rest assay, freshly emerged male flies were pre-entrained for 2 days in the LD
137 cycle at 25°C and at a humidity of 75 ± 5% inside the incubator (MIR-154, Panasonic, Japan).
138 Two-day-old virgin males were individually introduced into activity tubes. Flies were provided
139 with standard cornmeal medium and their activity/rest behavior was recorded using Drosophila
140 Activity Monitors (DAM, Trikinetics, USA). The infrared beam that passes through the middle
141 of the activity tubes count the movement of the flies. Following acclimatization in activity tubes
142 for 12 hours, flies were transferred to 1% agar medium or to standard cornmeal medium in glass
143 tubes at ZT12 to provide starved or fed conditions, respectively, and locomotor activity was
144 recorded for 36 hours. Each experiment was performed at least three times with 32 flies for each
145 genotype.

146 # Triglyceride assay

147 This assay comprised of three replicates and each replicate containing 5 male flies were
148 homogenized in homogenization buffer (0.05% Tween 20). The homogenate was incubated at
149 70° C for 5 minutes to inactivate the enzymes and centrifuged at 14000 rpm for 3 minutes and
150 the supernatant was collected into a sample tube. Sigma serum triglyceride determination kit
151 TR0100 was used to estimate the triglyceride level in the sample by measuring absorbance at
152 540 nm using TECAN Infinite M200 pro-multimode plate reader. The triglyceride content was

153 normalized to the total protein content of the flies estimated by Quick Start™ Bradford 1X Dye
154 Reagent (BioRad, Cat. # 500-0205). Assays were carried out at ZT 12 in freshly emerged (Day
155 0), 2 day old (Day 2) and 5 day old (Day 5) flies.

156 Starvation resistance Assay

157 Freshly emerged male flies (Day 0) were kept in the 1% agar medium vials. Six replicates
158 with each vial containing 10-15 flies were used for each genotype. The number of dead flies was
159 recorded at every two-hour interval until all the flies died.

160 Feeding assay

161 Two-day-old male flies were used for feeding assay. This assay comprised of three
162 replicates and each replicate containing 8 male flies were maintained under fed (corn medium)
163 and starved (1% agar medium) conditions for 24 hours starting from ZT00. After 24 hours of fed
164 and starved condition treatment, flies were provided with yeast paste mixed with Orange G
165 (Sigma) at ZT00 and allowed to feed for the next 30 minutes. In order to assess the amount of
166 food intake, flies were homogenized with 0.05% Tween 20 homogenization buffer and
167 zirconium oxide beads. The supernatant obtained after the centrifugation at 5000 rpm was used
168 for the calorimetric measurement at 478 nm using TECAN Infinite M200 pro multi-mode plate
169 reader. The absorbance measured from flies fed with normal food was subtracted from the
170 absorbance measured from the flies fed with orange G. The net absorbance reflected the amount
171 of food ingested.

172 Statistical analysis

173 Cosinor analysis was implemented in MATLAB-R2016a to test for rhythmicity, to
174 estimate the peak phase and the amplitude (peak/trough ratio) of relative mRNA expression of

175 *snpf* [31]. The waveform of activity/rest rhythm, total activity, day time activity, nighttime
176 activity and food intake was analyzed by using ANOVA followed by Tukey's post hoc multiple
177 comparisons. TGA levels and time taken for 50% of the fly death under starvation were
178 analyzed by Student's t-test. The statistical analyses were implemented on Statistica for
179 Windows Release 5.0 B [32]. Error bars in all the graphs represent standard error of the mean
180 (SEM).

181 Results

182 *snpf* transcript exhibit circadian oscillation

183 To assess whether the circadian clock regulates *snpf* transcript oscillation, the
184 transcription profile of *snpf* was estimated in w^{1118} flies under LD and under constant darkness
185 (DD). The *snpf* mRNA level increased during light phase and decreased during the dark phase
186 resulting in a significant daily oscillation with a peak at ZT 8.1h under LD (cosinor analysis,
187 $p<0.001$) (Fig 1A). The *snpf* transcriptional oscillation persisted under DD with a peak at CT
188 8.5h (cosinor analysis, $p<0.002$). However, the amplitude (peak/trough ratio) of the oscillation
189 decreased under DD compared to that obtained under LD (Fig 1B). These results suggest that
190 the transcriptional oscillation of *snpf* mRNA is endogenously controlled by the clock.

191 sNPF suppress starvation-mediated hyperactivity

192 To assess the role of sNPF in locomotor activity, we expressed $snpf^{RNAi}$ under *snpf-Gal4*
193 driver (*snpf-Gal4* > $snpf^{RNAi}$) and recorded the locomotor activity under fed LD conditions for 36
194 h. The *snpf-Gal4* > $snpf^{RNAi}$ flies exhibited increased activity around lights-on and after lights-
195 off compared to the control (*snpf-Gal4* > w^{1118}) flies (ANOVA, $p<0.0001$) (Fig 1C). This

196 resulted in enhanced total activity during day 1 (24 h) (ANOVA, $p<0.0001$) (Fig 1G). To test the
197 role of sNPF in starvation-mediated hyperactivity, we analyzed the locomotor activity of *snpf-*
198 *Gal4 > snpf* ^{RNAi} flies under fed and starved conditions and compared it with that of the control
199 flies. Both the control flies and the *snpf-Gal4 > snpf* ^{RNAi} flies exhibited an increase in total
200 activity on day 1 and during dark phase of day 2 (ZT 13-24) under starved condition compared to
201 what was observed under fed condition (*snpf-Gal4 > w*^{1118} flies; ANOVA, $p<0.0001$ for day1,
202 $p<0.0001$ for day2: ZT 13-24) (*snpf-Gal4 > snpf* ^{RNAi} flies; ANOVA, $p<0.0001$ for day1,
203 $p<0.0001$ for day2: ZT 13-24) (Fig 1D, E, G, H). Comparison of activity between control and
204 *snpf-Gal4 > snpf* ^{RNAi} flies under starved condition revealed that *snpf-Gal4 > snpf* ^{RNAi} flies
205 exhibited greater morning and evening activity peaks than control (Fig 1F), resulting in greater
206 total activity on Day1 (ANOVA, $p<0.01$) and during the dark phase of Day 2 (ZT 13-24)
207 (ANOVA, $p<0.0001$) (Fig 1 G, H). These results suggest that down regulation of sNPF in sNPF
208 expressing tissues enhances starvation-mediated hyperactivity in *Drosophila.*

209 We further assessed the role of sNPF in regulating the triglyceride levels and feeding
210 behavior. *Snpf-Gal4 > snpf* ^{RNAi} flies did not exhibit any significant difference in the starvation
211 resistance and triglyceride levels compared to the control flies (ANOVA, $p>0.05$) (Fig 1I, J). To
212 test whether sNPF play any role in hunger induced food intake after starvation, we examined the
213 food intake in both the control and the *snpf-Gal4 > snpf* ^{RNAi} flies under both fed and starvation.
214 Although starvation enhanced food intake (ANOVA, $p<0.0001$ for *snpf-Gal4 > w*^{1118}; ANOVA,
215 $p<0.0001$ for *snpf-Gal4 > snpf* ^{RNAi}) (Fig S1A), there was no significant difference in food intake
216 between the control and the *snpf-Gal4 > snpf* ^{RNAi} flies subjected to starvation, suggesting that
217 sNPF play no significant role in regulation of triglyceride level, starvation resistance and
218 enhanced food intake after starvation.

219

220 **Fig 1. Role of sNPF in starvation-mediated hyperactivity.** (A) Transcription profile of *snpf*
221 mRNA in w^{1118} flies under 12:12 h light-dark cycle (LD). White and grey shaded bars represent
222 light and dark phase, respectively, under LD. (B) Transcription profile of *snpf* mRNA in w^{1118}
223 flies constant darkness (DD). *snpf* transcript exhibited a significant daily oscillation with a peak
224 at ZT 8.1h under LD and the oscillation persisted under DD with a peak at CT 8.5h. (C) Average
225 locomotor activity counts/hour of *snpf-Gal4* > w^{1118} and *snpf-Gal4* > *UAS-snpf* ^{RNAi} (*snpf-Gal4* >
226 *snpf* ^{RNAi}) flies assayed with ad libitum food under LD for 36 h. Crossings per 1h are averaged
227 using 32 flies for each genotype. (D) Exposition of starvation-mediated hyperactivity by *snpf-*
228 *Gal4* > w^{1118} flies under starvation condition. Flies were transferred to fresh food or to 1% agar
229 medium at ZT12 to provide the fed and starved condition respectively. Gray shaded bars
230 indicate dark phase under LD for 36 h time window. Flies exhibited enhanced locomotor
231 activity under starvation condition compared to fed controls. (E) Average activity counts/hour of
232 *snpf-Gal4* > *snpf* ^{RNAi} flies assayed under fed and starved condition. (F) Exposition of starvation-
233 mediated hyperactivity by *snpf-Gal4* > w^{1118} and *snpf-Gal4* > *snpf* ^{RNAi} flies assayed in the
234 absence of food. (G, H) Total activity of *snpf-Gal4* > w^{1118} and *snpf-Gal4* > *snpf* ^{RNAi} flies on
235 Day1 (24h) and Day 2 (ZT13-24) under fed and starved conditions. Total activity (24h) is the
236 sum of activity exhibited under 12 hours of darkness and 12 hours of light. Starvation enhances
237 the activity in both control and experimental flies. Experimental flies exhibited a significant
238 increase in total activity compared to controls. (I) Percentage survivorship of freshly emerged
239 (Day 0) *snpf-Gal4* > w^{1118} and *snpf-Gal4* > *snpf* ^{RNAi} flies under starvation condition. (J)
240 Normalized triglyceride levels in Day 0, Day 2 and Day 5 old *snpf-Gal4* > w^{1118} and *snpf Gal4* >
241 *snpf* ^{RNAi} flies. No significant difference was observed in starvation resistance and triglyceride

242 levels. The asterisks on locomotor activity waveform and different lower case letters on the
243 vertical bar graphs indicate that the values are significantly different. Error bars represent mean ±
244 SEM. For further explanations see text.

245 sNPFR expression in IPC is required for starvation-mediated 246 hyperactivity

247 To test the role of sNPFR expressed in IPCs on starvation-mediated hyperactivity,
248 we expressed the *UAS-snpfR* ^{RNAi} under *dilp2-Gal4 GFP* driver (*dilp2-Gal4 GFP > snpfR* ^{RNAi}).
249 We recorded the locomotor activity of these flies under fed condition for 36 h and observed that
250 flies expressing *snpfR* ^{RNAi} in IPCs show an enhanced evening peak compared to the control flies
251 (ANOVA, $p<0.03$) (Fig 2A). We analyzed the effect of starvation on locomotor activity in the
252 control and the *dilp2-Gal4 GFP > snpfR* ^{RNAi} flies. When starved, the control flies exhibited an
253 increase in locomotor activity during the light phase of Day 1 (ANOVA, $p<0.001$) and during the
254 dark phase of Day 2 (ZT 13-24) (ANOVA, $p<0.003$) compared to those under fed condition (Fig
255 2B, F, G). We next examined whether sNPFR expressed in IPCs play any role in starvation-
256 mediated hyperactivity by comparing the locomotor activity of the *dilp2-Gal4 GFP > snpfR* ^{RNAi}
257 flies between fed and starved conditions. ANOVA revealed that starved *dilp2-Gal4 GFP >*
258 *snpfR* ^{RNAi} flies showed an enhanced evening peak and a prolonged morning peak compared to
259 the fed flies (ANOVA, $p<0.0001$) (Fig. 2C). This resulted in enhanced total activity during the
260 dark phase of Day 1 (ANOVA, $p<0.0001$) (Fig. 2E) and Day 2 (ZT 13-24) (ANOVA, $p<0.0001$)
261 (Fig. 2G). When compared the locomotor activity waveform between the control (*dilp2-Gal4*
262 *GFP >* w^{1118}) and the *dilp2-Gal4 GFP > snpfR* ^{RNAi} flies under starved condition, *dilp2-Gal4 >*
263 *snpfR* ^{RNAi} flies exhibited a greater evening peak on Day 1 and 2 (ANOVA, $p<0.001$) (Fig 2D),

264 showing greater total activity during the dark phase of Day 1 (ANOVA, $p<0.0001$) (Fig 2E) than
265 the control flies. In addition, the *dilp2-Gal4 GFP > snpfR* ^{RNAi} flies exhibited a marked decrease
266 in activity during the middle of the light phase on Day 1 (ANOVA, $p<0.001$) (Fig 2D, F),
267 suggesting that sNPF suppresses starvation-induced hyperactivity partly through the sNPFR
268 expressed in IPCs.

269 sNPF expression in circadian clock neurons is not required for 270 starvation-mediated hyperactivity

271 To test the role of sNPF expressed in circadian clock neurons on starvation-
272 mediated hyperactivity, we used flies expressing *UAS- snpf* ^{RNAi} under *tim-Gal4* driver (*tim-Gal4*
273 *> snpf* ^{RNAi}). Flies expressing *snpf* ^{RNAi} in clock neurons exhibited no significant differences in
274 starvation-mediated hyperactivity compared to the control flies (*tim-Gal4 >* w^{1118}) (Fig 2H),
275 suggesting that sNPF expressed in clock neurons has no significant effect on starvation-mediated
276 hyperactivity.

277

278 **Fig 2. Role of SNPF receptors expressed in IPC in regulating starvation-mediated**
279 **hyperactivity.** (A) Average activity counts/hour of *dilp2-Gal4 GFP >* w^{1118} and *dilp2-Gal4*
280 GFP > UAS-*snpfR* ^{RNAi} (*dilp2-Gal4* GFP > *snpfR* ^{RNAi}) flies assayed under the fed condition. (B)
281 Exposition of starvation-mediated hyperactivity by *dilp2-Gal4 GFP >* w^{1118} flies assayed in the
282 absence of food and compared it with their activity under fed condition. (C) Exposition of
283 starvation-mediated hyperactivity by *dilp2-Gal4 GFP > snpfR*^{RNAi} flies assayed in the absence of
284 food and compared it with their activity under the fed condition. (D) Starvation-mediated

285 hyperactivity of *dilp2-Gal4 GFP* > w^{1118} flies and *dilp2-Gal4 GFP* > $snpfR^{RNAi}$ flies assayed in
286 the absence of food. Experimental flies exhibited enhanced evening peak of activity compared
287 to the control flies under starved condition. (E, F) Total activity of *dilp2-Gal4 GFP* > w^{1118} and
288 *dilp2-Gal4 GFP* > *snpfR* ^{RNAi} flies during the dark phase and light phase of Day 1 under both fed
289 and starved conditions. (G) Total activity of *dilp2-Gal4 GFP* > w^{1118} and *dilp2-Gal4 GFP* >
290 *snpfR* ^{RNAi} flies during the dark phase of Day 2 under fed and starved conditions. (H) Exposition
291 of starvation-mediated hyperactivity by *tim-Gal4* > w^{1118} and *tim-Gal4* > *snpfR* ^{RNAi} flies assayed
292 in the absence of food. Other details are the same as in Fig 1.

293 Role of *dilp2* in starvation-mediated hyperactivity

294 As it is already known that sNPF regulates *dilp2* expression in IPCs, we further
295 studied the role of *dilp2* in starvation-mediated hyperactivity. To examine the effect of tissue-
296 specific knockdown of *dilp2* in IPCs on starvation-mediated hyperactivity, we expressed the
297 *UAS-dilp2* ^{RNAi} under *dilp2-Gal4* driver (*dilp2-Gal4* > *dilp2* ^{RNAi}). We recorded the locomotor
298 activity of the *dilp2-Gal4* > *dilp2* ^{RNAi} flies expressing *dilp2* ^{RNAi} in IPCs under the fed condition.
299 These flies exhibited a reduction in the light phase activity of Day 1 compared to controls
300 (ANOVA, p<0.0001) (Fig 3A). To test the effect of starvation on the locomotor activity, we
301 analyzed the locomotor activity of control (*dilp2-Gal4* > w^{1118}) flies under fed and starved
302 conditions. The starved control flies exhibited an increased activity during the light phase of
303 Day 1 (ANOVA, p<0.0001) (Fig 3B), resulting in enhanced total activity on Day 1 (24 h)
304 (ANOVA, p<0.001) (Fig 3E) compared to fed flies. We next examined whether *dilp2* plays any
305 role in starvation-mediated hyperactivity by recording the locomotor activity of the *dilp2-Gal4* >
306 *dilp2* ^{RNAi} flies under fed and starved conditions. Although the starved *dilp2-Gal4* > *dilp2* ^{RNAi}

307 flies showed enhanced daytime activity during the first day of starvation (Fig 3B), there was no
308 significant changes in daily total activity on Day 1 compared to the fed flies (ANOVA, $p>0.05$)
309 (Fig 3E). During the 2nd dark phase, however, the starved flies exhibited an aberrant and
310 enhanced activity (ANOVA, $p<0.0001$) (Fig 3C, F). Comparison between the starved control
311 and *dilp2-Gal4* > *dilp2* ^{RNAi} flies revealed that *dilp2-Gal4* > *dilp2* ^{RNAi} flies exhibited less total
312 activity than control flies on Day 1 (ANOVA, p<0.0001) (Fig 3D, E). However, there were no
313 significant differences in total activity on Day 1 between the *dilp2-Gal4* > *dilp2* ^{RNAi} flies under
314 both fed and starved conditions (Fig 3E). These results suggest that down regulation of *dilp2* in
315 IPCs reduces the starvation-mediated hyperactivity only during the first 24 h of starvation.

316 *dilp2* governs starvation resistance and triglyceride metabolism

317 We further assessed the starvation resistance of freshly emerged *dilp2-Gal4* > *dilp2*^{RNAi}
318 flies and compared it with that of the control (*dilp2-Gal4* > w^{1118}) flies. The *dilp2-Gal4* > *dilp2*
319 ^{RNAi} flies showed reduced starvation resistance compared to the control flies (t-test, $p<0.03$) (Fig
320 3G, S1B). We quantified triglyceride levels on Day 0, Day 2 and Day 5 in *dilp2-Gal4* > w^{1118}
321 and *dilp2-Gal4* > *dilp2*^{RNAi} flies and found that the triglyceride levels were significantly reduced
322 in *dilp2-Gal4* > *dilp2* ^{RNAi} flies on these days (t-test, p<0.0001 for Day 0, t-test, p<0.02 for Day 2,
323 t-test, p<0.0001 for Day 5) (Fig 3H). These results suggest that *dilp2* regulates starvation
324 resistance by maintaining the triglyceride metabolism.

325

326 **Fig 3. Role of *dilp2* in starvation-mediated hyperactivity and triglyceride metabolism.** (A)
327 Average locomotor activity counts/hour of *dilp2-Gal4* > w^{1118} and *dilp2-Gal4* > *UAS-dilp2* ^{RNAi}

328 *(dilp2-Gal4 > dilp2* ^{RNAi}) flies assayed under the fed condition. (B) Exposition of starvation-
329 mediated hyperactivity by *dilp2-Gal4 > w*^{1118} flies assayed in the absence of food and compared
330 it with their activity under the fed condition. (C) Exposition of starvation-mediated hyperactivity
331 by *dilp2-Gal4 > dilp2* ^{RNAi} flies assayed under starvation condition and compared it with their
332 activity under the fed condition. (D) Starvation-mediated hyperactivity of *dilp2-Gal4* control
333 flies and *dilp2-Gal4 > dilp2*^{RNAi} flies assayed in the absence of food. Experimental flies
334 exhibited a reduction in activity during the light phase of day 1 compared to controls under
335 starvation condition. (E, F) Total activity (24 h) of *dilp2-Gal4 > w*^{1118} and *dilp2-Gal4 > dilp2*
336 ^{RNAi} flies on Day1 and during the dark phase (ZT 13-24 hr) of Day 2 under fed and starved
337 conditions. *dilp2-Gal4 > dilp2* ^{RNAi} flies exhibited a reduction in total activity on Day 1
338 compared to *dilp2-Gal4 > w*^{1118} flies under starvation condition, whereas *dilp2-Gal4 > dilp2* ^{RNAi}
339 flies did not exhibit any significant difference in total activity on Day 1 under both fed and
340 starved conditions. (G) Percentage survivorship of freshly emerged flies expressing *dilp2*^{RNAi} in
341 IPC under *dilp2-Gal4* control under starvation condition. Flies expressing *dilp2*^{RNAi} in IPCs
342 exhibit reduced starvation resistance compared to the control flies. (H) Normalized triglyceride
343 levels of Day 0, Day 2 and Day 5 old *dilp2-Gal4 > w*^{1118} and *dilp2-Gal4 > dilp2* ^{RNAi} flies. Flies
344 expressing *dilp2* ^{RNAi} in IPCs exhibit reduced triglyceride levels compared to the control flies.
345 Other details are the same as in Fig 1.

346 Role of insulin receptors expressed in the fat body in regulating 347 starvation-mediated hyperactivity.

348 Since *dilp2* is shown to effect fat metabolism, we checked for the effects by disrupting
349 the insulin signalling in the fat body, the tissue responsible for the storage of lipid droplets. To

assess whether insulin receptors (*InR*) expressed in the fat body play any role in regulating the starvation-mediated hyperactivity, we overexpressed *InR* dominant negative (InR^{DN}) under the *CG-Gal4* driver (*CG-Gal4* > InR^{DN}) and recorded the locomotor activity of the *CG Gal4* > InR^{DN} flies. The *CG Gal4* > InR^{DN} flies overexpressing InR^{DN} in fat body exhibited reduced activity during the middle of the light phase under the fed condition compared to the control (*CG-Gal4* > w^{1118}) flies (ANOVA, $p<0.0001$) (Fig 4A). To test the effect of insulin receptors (*InR*) expressed in the fat body in starvation-mediated hyperactivity, we recorded the locomotor activity of control and *CG-Gal4* > InR^{DN} flies under fed and starved conditions. The starved control flies exhibited an increase in locomotion during the light phase on Day 1 (ANOVA, $p<0.006$) (Fig 4B, F) and during the dark phase of Day 2 (ZT 13-24) compared to fed flies (ANOVA, $p<0.0001$) (Fig 4B, G). Starved *CG-Gal4* > InR^{DN} flies showed enhanced daytime activity compared to the fed flies on Day1 (ANOVA, $p<0.0001$) (Fig 4C). The starved flies exhibited aberrant and enhanced activity during the 2nd dark phase (ANOVA, $p<0.0001$) (Fig 4C, G). Comparison the starvation induced hyperactivity between control and *CG-Gal4* > InR^{DN} flies revealed that *CG-Gal4* > InR^{DN} flies exhibited a greater evening and morning peaks but less activity during the middle of dark and light phase on day 1 (ANOVA, $p<0.04$) (Fig 4D) and that they showed greater activity during the 2nd dark phase. These results suggest that down regulation of *InR* in the fat body alters the starvation-mediated hyperactivity.

Involvement of *InR* in starvation resistance and triglyceride metabolism

We further compared the starvation resistance between freshly emerged (Day 0) *CG-Gal4* > InR^{DN} flies and *CG-Gal4* > w^{1118} control flies. The *CG-Gal4* > InR^{DN} flies showed

372 reduced starvation resistance compared to the control flies (t-test, $p<0.04$) (Fig 4H, I).
373 Consistent with the starvation resistance phenotype, they showed decreased triglyceride levels (t-
374 test, $p<0.003$) (Fig 4J), suggesting involvement of *InR* expressed in the fat body in starvation
375 resistance and triglyceride metabolism.

376 *InR* expressed in fat body governs enhanced food intake after 377 starvation

378 We next compared the food intake between *CG-Gal4* > InR^{DN} flies and control (*CG-*
379 *Gal4* > w^{1118}) flies. The results showed that 24h starvation enhances food intake in control flies
380 (ANOVA, $p< 0.03$). Food intake was more enhanced in starved *CG-Gal4* > InR^{DN} flies
381 compared to the starved control flies (ANOVA, $p<0.01$) (Fig S1C), suggesting that insulin
382 signaling in fat body suppresses food intake under starvation.

383

384 **Fig 4. Role of insulin receptors (*InR*) expressed in the fat body in regulating starvation-**
385 **mediated hyperactivity and triglyceride metabolism.** (A) Average activity counts/hour of
386 *CG-Gal4* > w^{1118} and *CG-Gal4* > *UAS-InR* ^{DN} (*CG-Gal4* > InR^{DN}) flies assayed under fed
387 condition. (B) Exposition of starvation-mediated hyperactivity by *CG-Gal4* > w^{1118} flies assayed
388 under starvation condition and compared it with their activity under the fed condition. (C)
389 Exposition of starvation-mediated hyperactivity by *CG-Gal4* > InR^{DN} flies assayed in the
390 absence of food and compared it with their activity under fed condition. (D) Locomotor activity
391 waveform of *CG-Gal4* > w^{1118} control flies and *CG-Gal4* > InR^{DN} flies assayed under starvation
392 condition. Flies expressing InR^{DN} in the fat body alter starvation-mediated hyperactivity

393 compared to control flies. (E, F) Total activity of *CG-Gal4* > w^{1118} and *CG-Gal4* > *InR* ^{DN} flies
394 during the dark phase and light phase of Day 1 under fed and starved conditions. (G) Total
395 activity of *CG- Gal4* > w^{1118} and *CG-Gal4* > *InR* ^{DN} flies during the dark phase of Day 2 under
396 fed and starved conditions. (H) Percentage survivorship of freshly emerged *CG- Gal4* > *InR* ^{DN}
397 and control flies under starvation condition. (I) Time taken for 50% of death in *CG-Gal4* > w^{1118}
398 control and *CG-Gal4* > *InR* ^{DN} flies under starvation condition. Expressing *InR* ^{DN} in the fat body
399 reduced the starvation resistance in freshly emerged flies. (J) Triglyceride content of freshly
400 emerged (Day 0) *CG*-Gal4 > w^{1118} control and *CG-Gal4* > *InR* ^{DN} flies. Expressing *InR* ^{DN} in the
401 fat body reduced the triglyceride levels in freshly emerged flies. Other details are the same as in
402 Fig 1.

403 Discussion

404 Behavior and metabolism are functionally intertwined processes that modulate each
405 other. However, it remains elusive how the circadian clock governs behaviors, including feeding
406 and locomotor activity, in synchrony with the metabolic status of an organism. In the present
407 study, we aimed to investigate the specific role of two circadian relevant metabolic
408 neuropeptides, i.e., sNPF and DILP2, in lipid metabolism and starvation-mediated behavioral
409 changes in *Drosophila*. The results of the present study showed that *snpf* transcript exhibited a
410 circadian oscillation in the fly head. sNPF protein is expressed in a diverse subset of neurons in
411 the adult fly brain including olfactory receptor neurons, various small interneurons, kenyon cells
412 of the mushroom bodies and in the circadian clock neurons [33]. Among the ~150 clock neurons
413 clustered in the fly brain, two dorsal lateral neurons (LNds) and PDF expressing small ventral
414 lateral neurons (s-LNvs) express sNPF [34]. Based on the previous findings, mRNA cycling is

415 essential for the circadian clock neuron function including the neuronal firing or electrical
416 excitability [25], [35]. Hence it is quite likely that the *snpf* transcript oscillation is more apparent
417 in circadian clock neurons than other sNPF expressing diverse neuronal subsets. Most of the
418 previously identified cycling head transcripts are tissue or cell-specific in the mammalian system
419 [36]. Ubiquitous mRNA cycling is relatively rare. Further studies are required to confirm
420 whether sNPF oscillation is indeed restricted to clock neurons or if it also oscillates in non-clock
421 neurons.

422 sNPF expression in a large population of diverse neurons indicates the multiple discrete
423 functions of this neuropeptide [33]. So far studies demonstrated that sNPF regulates feeding and
424 its expression in fan shaped body might be involved in fine-tuning the locomotor activity level
425 [24], [37]. Expression of sNPF and sNPF receptor in the mushroom body and clock neurons
426 regulate sleep in *Drosophila* [25], [38]. The results of our present studies showed that
427 knockdown of *snpf* in sNPF expressing neurons enhances starvation-mediated hyperactivity,
428 suggesting the role of sNPF in suppressing hyperactivity under starvation. sNPF acts as an
429 inhibitory modulator in neuronal networks that express sNPFR [39]. Knockdown of *snpfR* in
430 IPCs in an adult stage-specific manner does not affect the starvation-mediated sleep loss in
431 *Drosophila* [24], whereas the results of our study showed that constitutive knockdown of *snpfR*
432 in IPCs partially suppress starvation-mediated hyperactivity in *Drosophila*. sNPF may suppress
433 the starvation-mediated hyperactivity mainly via IPCs not through the circadian clock neurons in
434 *Drosophila*. Our results showed that sNPF play no significant role in regulation of triglyceride
435 levels and starvation-mediated feeding behavioral changes suggesting that sNPF independently
436 regulate starvation-induced hyperactivity without an effect on enhanced food intake upon
437 starvation.

438 IPCs are metabolic clock output cells and the rhythmic output of IPC is modulated
439 by both the circadian clock and nutrient uptake [19]. Flies expressing *dilp2* RNAi in IPCs and
440 flies expressing InR^{DN} in the fat body exhibit differential starvation-induced hyperactivity
441 phenotypes. Since, in the latter, InR was blocked only in the fat body, InR expression in other
442 tissues may partly regulate the starvation-mediated hyperactivity phenotype [29]. This could be
443 the possible reason for the difference observed in the starvation-mediated hyperactivity
444 phenotypes between the flies expressing *dilp2*-RNAi in IPCs and the flies expressing InR^{DN} in
445 the fat body. Our results showed for the first time that the clock output molecule DILP2 plays an
446 important role in starvation resistance, and triglyceride metabolism in *Drosophila*. It has been
447 documented that the peripheral circadian clock in the *Drosophila* fat body regulates the storage
448 of lipid and glycogen [5], [16]. However, the potential mediators through which the central
449 clock communicates with the peripheral clock to regulate the triglyceride metabolism still
450 remains to be elucidated. In the present study, flies expressing *dilp2* RNAi in IPCs and flies
451 expressing InR^{DN} in the fat body have reduced starvation resistance and triglyceride levels,
452 suggesting the role of DILP2 as a potential mediator involved in triglyceride metabolism.

453 *Drosophila* insulin like peptide2 (DILP2) drives rhythmicity in the expression of
454 lipase *sxe2* transcript in the fat body. Rhythmic expression of *sxe2* is disrupted in flies
455 expressing dominant negative insulin receptor InR^{DN} in the fat body [19]. Hence it is likely that
456 perturbation on *sxe2* oscillation plays a role in the reducing the triglyceride levels in flies
457 expressing *dilp2*-RNAi in IPCs and the flies expressing InR^{DN} in the fat body. Further studies are
458 required to confirm whether DILP2 drive the oscillation of other fat body specific gene
459 transcript/s to mediate triglyceride metabolism. Reduction in triglyceride level could be either
460 due to the enhanced energy expenditure or due to reduced energy intake [40]. We also tested

461 whether reduced triglyceride level is due to the reduced energy intake. Our results showed that
462 expressing InR^{DN} in the fat body does not have significant impact on food intake under fed
463 condition whereas these flies exhibit enhanced food intake only under starved condition. It is
464 thus also likely that the insulin signaling may play a role in suppressing food intake only under
465 starved conditions not under fed condition. In the present study, since the food intake under
466 starvation condition was assessed only at ZT00 after 24 h of starvation, we need further evidence
467 to confirm whether hyperactivity exhibited as a response to starvation is indeed food-seeking
468 behavior.

469 Taken together, our present study showed that sNPF suppresses the starvation-
470 mediated hyperactivity partially through sNPF receptors expressed in the IPCs. The insulin
471 signaling mediated by the circadian clock output molecule DILP2 in the fat body appears to be a
472 central pathway in triglyceride metabolism and regulates feeding behavior in accordance with the
473 nutrient availability in *Drosophila*.

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481

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610 # Supporting information

611 **S1 Fig. Role of sNPF and DILP2 in feeding behavior after starvation.** (A) Food intake in 2-
612 day-old *snpf-Gal4* > w^{1118} control and *snpf-Gal4* > *snpf* ^{RNAi} flies under fed and starved
613 conditions. Both control and experimental flies exhibited enhanced food intake after 24hr of
614 starvation compared to control flies under fed condition. (B) Time taken for 50% death of
615 freshly emerged flies expressing *dilp2*^{RNAi} in IPC under *dilp2-Gal4* > w^{1118} control under
616 starvation condition. (C) Food intake in 2-day-old *CG-Gal4* > w^{1118} control and *CG-Gal4* > *InR*
617 ^{DN} flies under fed and starved conditions. Experimental flies exhibited enhanced food intake
618 after 24h of starvation compared to starved *CG-Gal4* > w^{1118} flies.

Fig 1

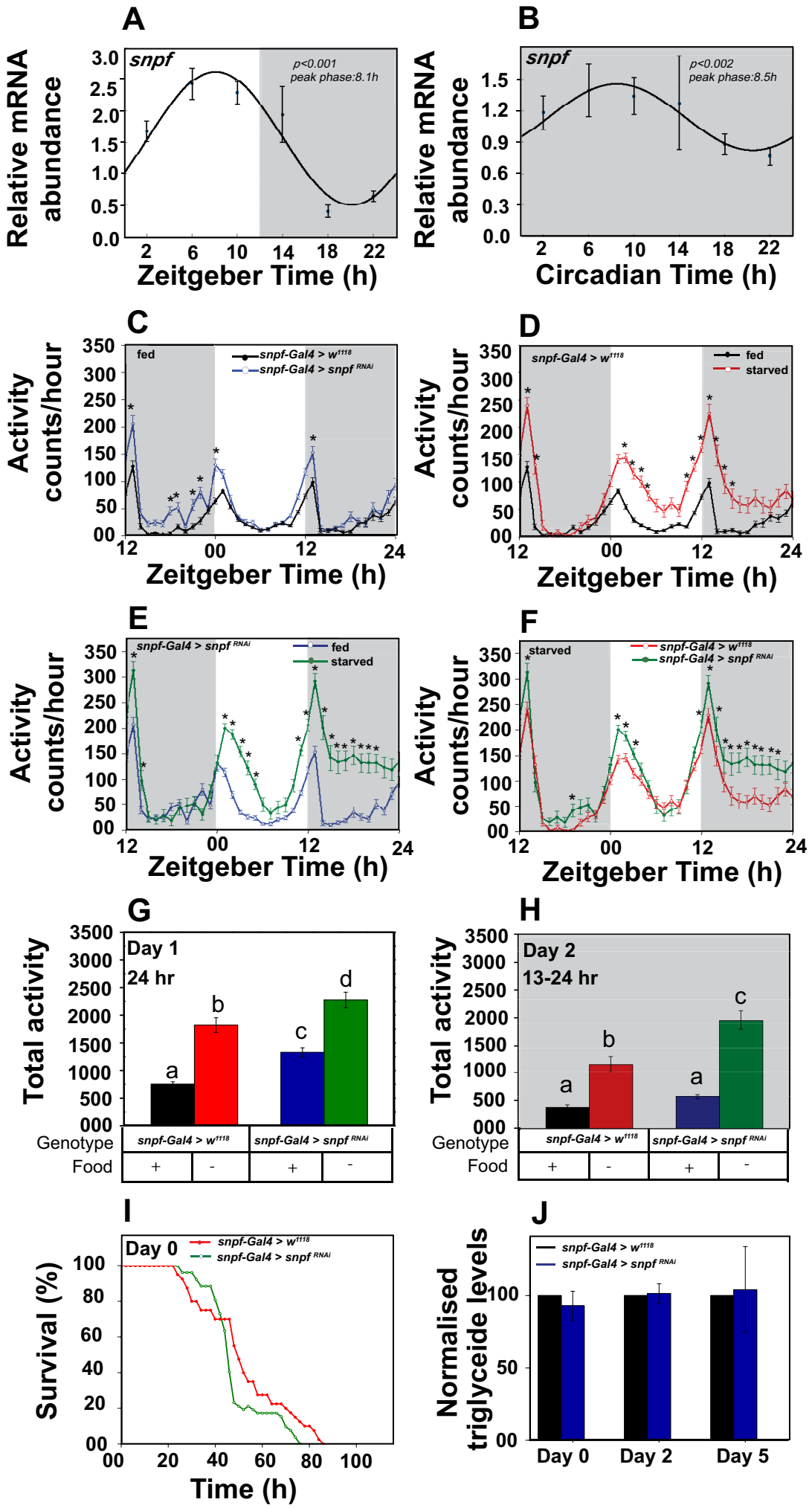
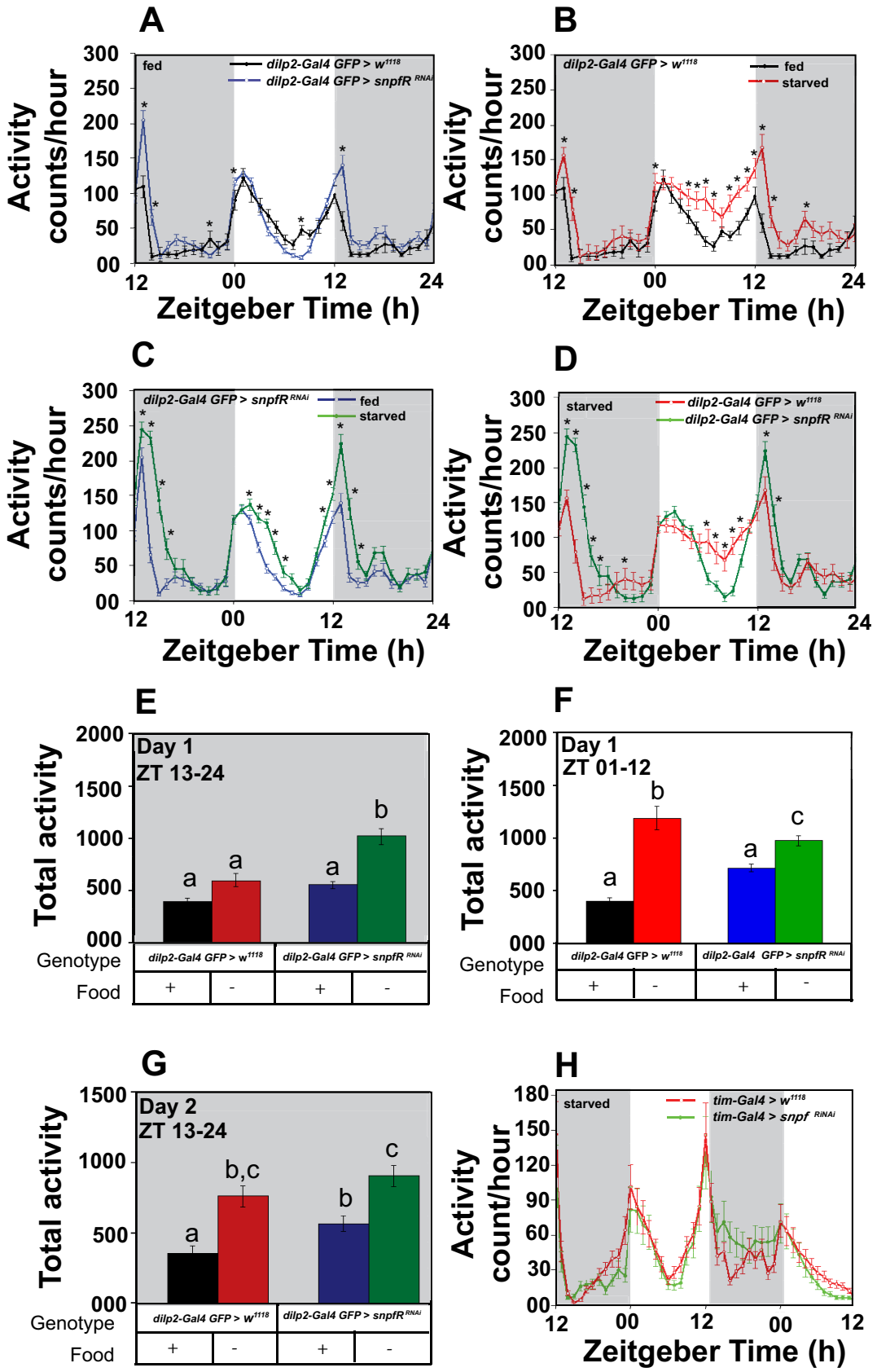

A
snpf
p<0.001
peak phase:8.1h
Relative mRNA abundance
Zeitgeber Time (h)
B
snpf
p<0.002
peak phase:8.5h
Relative mRNA abundance
Circadian Time (h)
C
fed
snpf-Gal4 > w1118
snpf-Gal4 > snpf RNAi
Activity counts/hour
Zeitgeber Time (h)
D
snpf-Gal4 > w1118
fed
starved
Activity counts/hour
Zeitgeber Time (h)
E
snpf-Gal4 > snpf RNAi
fed
starved
Activity counts/hour
Zeitgeber Time (h)
F
starved
snpf-Gal4 > w1118
snpf-Gal4 > snpf RNAi
Activity counts/hour
Zeitgeber Time (h)
G
Day 1
24 hr
Total activity
Genotype
Food
snpf-Gal4 > w1118
snpf-Gal4 > snpf RNAi
H
Day 2
13-24 hr
Total activity
Genotype
Food
snpf-Gal4 > w1118
snpf-Gal4 > snpf RNAi
I
Day 0
snpf-Gal4 > w1118
snpf-Gal4 > snpf RNAi
Survival (%)
Time (h)
J
snpf-Gal4 > w1118
snpf-Gal4 > snpf RNAi
Normalised triglyceide levels
Day 0
Day 2
Day 5

Fig 2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fed
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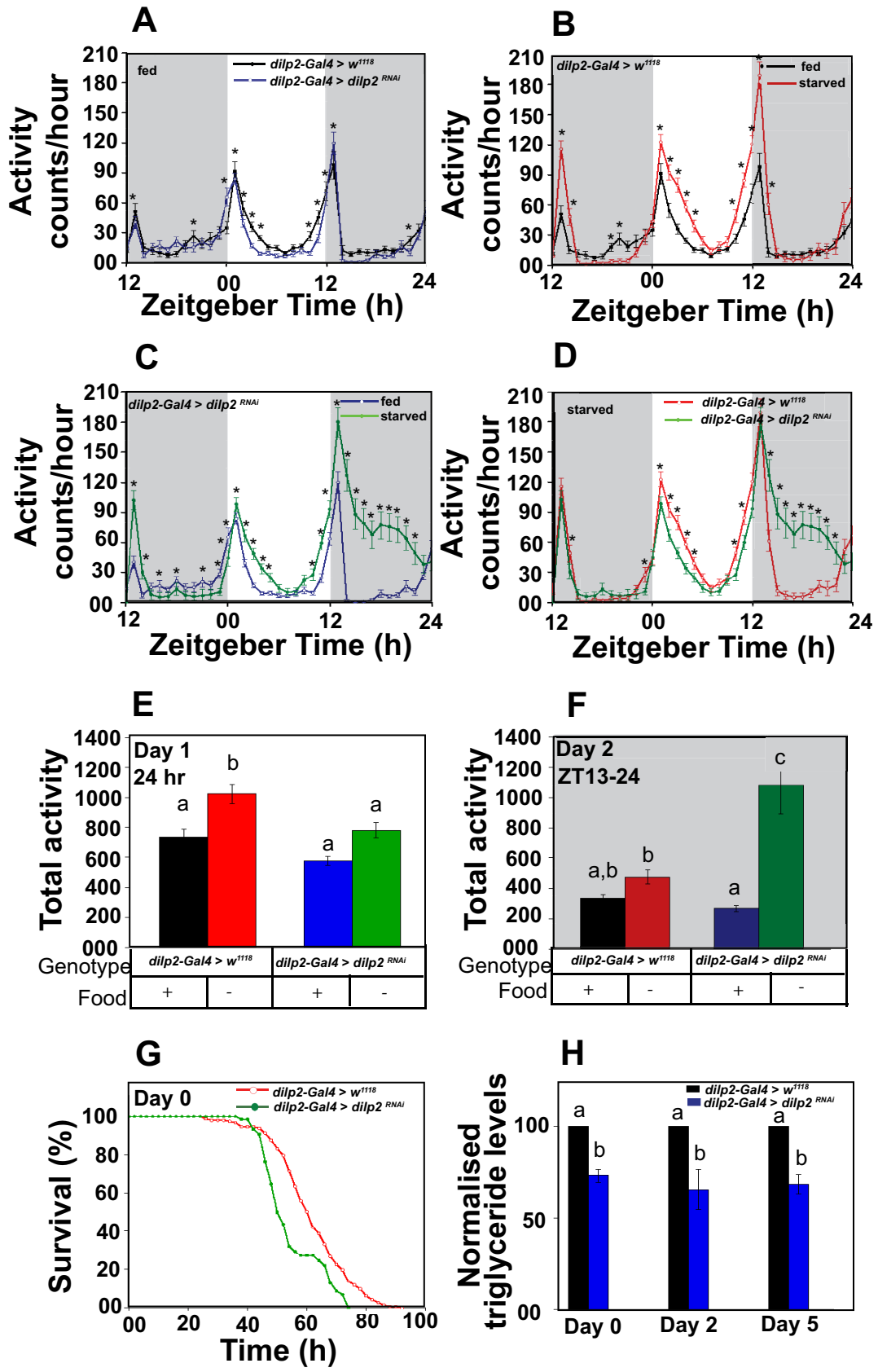
dilp2-Gal4 GFP > snpfR RNAi
Activity counts/hour
Zeitgeber Time (h)
B
dilp2-Gal4 GFP > w1118
fed
starved
C
dilp2-Gal4 GFP > snpfR RNAi
D
starved
E
Day 1
ZT 13-24
Total activity
Genotype
Food
F
Day 1
ZT 01-12
G
Day 2
ZT 13-24
H
tim-Gal4 > w1118
tim-Gal4 > snpf RINAi
Activity count/hour

Fig 3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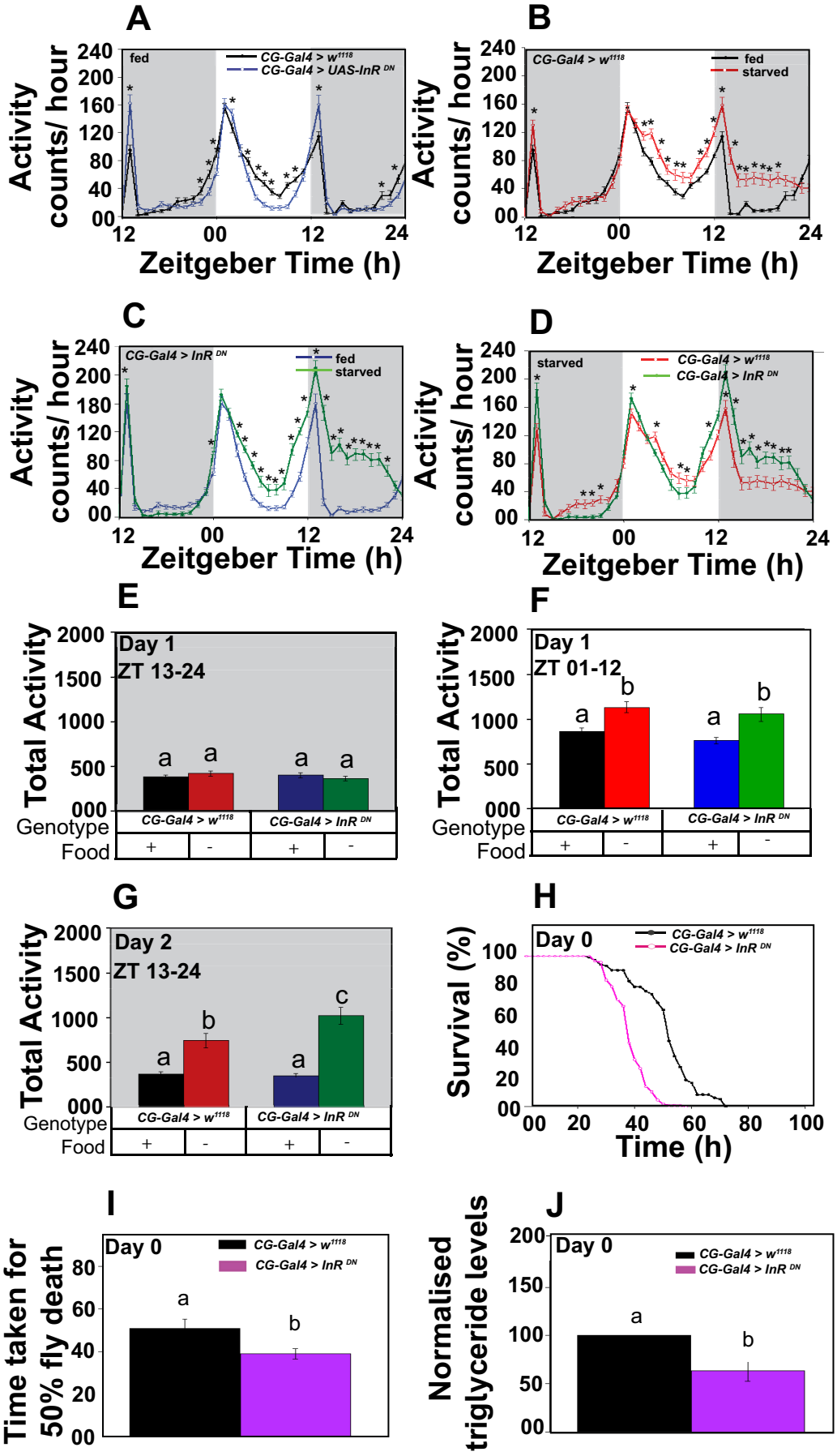
fed
dilp2-Gal4 > w1118
dilp2-Gal4 > dilp2 RNAi
Activity counts/hour
Zeitgeber Time (h)
B
dilp2-Gal4 > w1118
fed
starved
C
dilp2-Gal4 > dilp2 RNAi
D
starved
E
Day 1
24 hr
Total activity
Genotype
Food
F
Day 2
ZT13-24
G
Day 0
Survival (%)
Time (h)
H
Normalised triglyceride levels
Day 0
Day 2
Day 5

Fig 4


A
fed
CG-Gal4 > w1118
CG-Gal4 > UAS-InR DN
Activity counts/ hour
Zeitgeber Time (h)
B
CG-Gal4 > w1118
fed
starved
C
CG-Gal4 > InR DN
D
E
Day 1
ZT 13-24
Total Activity
Genotype
Food
F
Day 1
ZT 01-12
G
Day 2
ZT 13-24
H
Day 0
Survival (%)
Time (h)
I
Time taken for 50% fly death
J
Normalised triglyceride levels