Title: Deciphering a beetle clock: individual and sex-dependent variation in daily activity patterns

Running title: Locomotor activity rhythms in *T. castaneum*

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Abstract

Circadian clocks are inherent to most organisms, including cryptozoic animals that seldom encounter direct light, and regulate their daily activity cycles. A conserved suite of clock genes underpins these rhythms. In this study, we explore the circadian behaviors of the red flour beetle *Tribolium castaneum*, a significant pest impacting stored grain globally. We report on how daily light and temperature cues synchronize distinct activity patterns in these beetles, characterized by reduced morning activity and increased evening activity, anticipating the respective environmental transitions. Although less robust, rhythmicity in locomotor activity is maintained in constant dark and constant light conditions. Notably, we observed more robust rhythmic behaviors in males than females with individual variations exceeding those previously reported for other insect species. RNA interference targeting the *Clock* gene disrupted locomotor activity rhythms. Our findings demonstrate the existence of a circadian clock and of clock-controlled behaviors in *T. castaneum*. Furthermore, they highlight substantial individual differences in circadian activity, laying the groundwork for future research on

34 the relevance of individual variation in circadian rhythms in an ecological and
35 evolutionary context.

36 **Introduction**

37 Virtually all lifeforms from bacteria to humans express daily patterns in their
38 physiological and behavioral processes, known as circadian rhythms, which enable them
39 to accommodate the recurring changes in their environment (Saunders, 2002). These
40 rhythms are not only reactions to the environment but are endogenously controlled by
41 molecular pacemaker systems called circadian clocks, which can be entrained to the
42 external periodic cues such as light and temperature, which are collectively known as
43 zeitgebers (Bell-Pedersen et al., 2005). Coordinating activities in anticipation to the
44 periodic environmental changes has obvious adaptive functions such as escaping
45 adverse environmental conditions, ensuring proper availability of food and mates,
46 synchronizing behaviours with conspecifics and reducing competition or predation risks
47 (Favreau et al., 2009; Vaze and Sharma, 2013). Apart from these extrinsic advantages, a
48 circadian clock confers an intrinsic advantage of maintaining a stable and optimal
49 temporal compartmentalization of biological processes regardless of any irregular
50 fluctuations in the environment. Even animals living in aperiodic conditions are known
51 to have a functioning molecular clock (Trajano and Menna-Barreto, 1996) which points
52 towards its intrinsic adaptive values.

53 Circadian clocks operate similarly across different taxa. Their cellular mechanism
54 is comprised of transcription-translation feedback loops of clock genes and their protein
55 products, capable of producing self-sustained circadian oscillations (Doležel, 2023;
56 Helfrich-Förster, 2004). Detailed knowledge about the molecular mechanisms of animal
57 clocks is available mainly from the studies in mouse and fruit fly as representatives of
58 mammals and insects, respectively. In the main circadian feedback loop of fruit flies, the
59 clock proteins PERIOD (PER) and TIMELESS (TIM) represent the transcriptional
60 repressors, whereas in mammals CRYPTOCHOROMES (CRY1 and CRY2) dimerize with the
61 PERIOD proteins (mPER1, mPER2 and mPER3) to perform the equivalent role. This
62 transcriptional inhibition occurs by blocking of their transcriptional activators CLOCK and
63 CYCLE (BMAL1 is the mouse homolog of CYCLE). A striking difference between the
64 mammalian and *Drosophila* clock lies in the mechanism by which light resets and
65 synchronizes the clock with the environment. Light induces TIM degradation in flies
66 whereas it leads to the transcriptional activation of the *Period* genes in mice (Helfrich-
67 Förster, 2004).
68 Insect clocks are best documented in *Drosophila*. However, given the enormous
69 diversity of insect species and their habitats, it is plausible that their molecular clock
70 mechanisms have diversified in response to their specific environmental conditions
71 (Kotwica-Rolinska et al., 2022; Tomioka and Matsumoto, 2015). Diversities in some clock

72 components are already known, for instance, certain insects like beetles and honeybees
73 possess the mammalian light-insensitive cryptochrome, CRY2, unlike *Drosophila*, which
74 possesses only the photosensitive CRY1. On the other hand, mosquitoes and monarch
75 butterflies are known to possess both CRY1 and CRY2 (Yuan et al., 2007). This
76 underscores the significance of investigating a broad range of insect models for gaining
77 a comprehensive understanding of various aspects of the circadian clock.

78 *Tribolium castaneum,* the red flour beetle, is a widely found pest of stored grains
79 and an important model organism of increasing relevance in many fields such as classical
80 genetics, population genetics, insect development and epigenetics (Campbell et al.,
81 2022). These flour beetles are cryptozoic, meaning that they are animals living mostly in
82 darkness (Brown et al., 2009) They have very little exposure to light in their habitat of
83 stored grains or behind tree barks, their supposed original environment (Good, 1936;
84 Sokoloff, 1974)**.** Their cryptozoic adaptations include loss of photoreceptors sensitive to
85 blue light (Jackowska et al., 2007) and an increase in odorant and gustatory receptors
86 (Richards et al., 2008). Even though the adults are normally known for being negatively
87 phototactic (Park, 1934), they crawl to flour surfaces from time to time where they can
88 get direct light exposure as observed in laboratory populations (Misra and Englert,
89 1985). Copulation usually occurs on the flour surface as well (Park, 1934). They are also
90 likely to experience temperature variations in their natural habitats. Despite spending

91 most of their life in the absence of light, we know that they still possess the genes of a
92 functional molecular clock (Li et al., 2018). However, whether *T. castaneum* indeed
93 displays rhythmic activity profiles, driven by a functional circadian clock, has not been
94 convincingly demonstrated.

95 *T. castaneum* has homologs for all clock genes, known in *D. melanogaster*
96 (Richards et al., 2008). First studies on the molecular clock in *T. castaneum* revealed that
97 *Clock* (*Clk*), *cycle* (*cyc*), *period* (*per*) and *timeless* (*tim*) are all rhythmically expressed (Li
98 et al., 2018). Instead of the *Drosophila*-type cryptochrome, CRY1, these beetles possess
99 the light-insensitive CRY2, the function of which remains unclear. It has been suggested
100 that CRY2 plays a role in inhibiting CLK and CYC, thus acting as a negative element of the
101 feedback loop as known in other insects such as Hymenoptera and Heteroptera
102 (Kotwica-Rolinska et al., 2022; Yuan et al., 2007). However, *T. castaneum* also possesses
103 the *Drosophila*-type TIM (Kotwica-Rolinska et al., 2022) along with the mammalian CRY2
104 making it a unique model to study insect clocks, also possibly in an evolutionary context
105 of transition from the *Drosophila*-type to mammalian-type clocks.

106 In insects, functional circadian clocks, and the rhythms they produce are
107 traditionally evaluated by measuring an individual's variation in locomotor activity
108 during the course of the day (Chiu et al., 2010). It has been shown that *T.castaneum*
109 exhibits rhythmic locomotor activity under light dark cycles (LD; Rath et al., 2021) as well

110 as in the absence of external cues in constant darkness (DD; Abe et al., 2021). However,
111 both studies report only average data of individually kept beetles, without determining
112 the overall rhythmicity (i.e., how many beetles behaved rhythmically in a certain
113 condition), nor showing individual actograms, which would allow to estimate the
114 individual variation within the population. It is important to investigate these behavioral
115 variations as an organism reacts to and modifies its environment through its behavior
116 (Duckworth, 2009) and therefore having sufficient behavioral variation can readily help
117 populations to adapt faster to changing environments (Bolnick et al., 2003).
118 Furthermore, to our knowledge, there are also no studies exploring whether the
119 circadian clock in these beetles can be synchronized by temperature fluctuations.

120 In the present study, we monitored the locomotor activity patterns of *T.*
121 *castaneum* to characterize in detail the circadian profiles in their movements. We
122 studied light as well as temperature entrainment and the effect of constant conditions
123 on an individual level to characterise the behavioral variations existing within the
124 population. We also performed RNAi to study the changes in activity patterns upon
125 knockdown of the clock gene encoding the key circadian transcription factor CLK. As *T.*
126 *castaneum* is an important model organism, knowledge about their biological clock and
127 how it influences their behavior and physiology can add to the knowledge in their proper
128 laboratory rearing conditions and proper timing of experiments. It can serve as an

129 exceptional model system for investigating the molecular clock mechanisms of
130 Coleoptera, where our current understanding is comparatively limited and thereby also
131 advancing our overall comprehension of insect clocks.

132 **Materials and Methods**

133 **Beetle cultures**

134 For this study, the Croatia 1 (Cro1) strain, collected in Croatia in 2010 and a
135 commonly used lab-adapted strain San Bernardino (SB), from San Bernadino, California,
136 USA, established by Sokoloff in 1960's were used (Milutinović et al., 2013). The
137 populations were kept at standard laboratory conditions of 30 ºC, 70% humidity and a
138 12 h:12 h light: dark cycle (lights on at 08:00 and lights off at 20:00; light from
139 flourescent bulbs, 4000 K, cool white, 3350 lumen) in plastic boxes or glass jars with
140 foam stoppers to allow air circulation. The standard beetle diet consisted of heat-
141 sterilized (75 ºC for at least 24 h) organic wheat flour (Bio Weizenmehl Type 550, dm -
142 Drogerie Market) mixed with 5% brewer's yeast powder (referred to as "flour" from now
143 on).

144 **Locomotor activity**

145 To monitor locomotor activity patterns, we used Drosophila Activity Monitoring
146 System (DAM5H-4, Trikinetics Inc., Waltham, MA, USA; referred to as "DAM5 system" in

147 the following). Individual beetle's movements interrupt one of four infrared light beams
148 intersecting the glass tubes housing single beetles, at equal distances from each other
149 (Rosato and Kyriacou, 2006, Fig S1) which enables the recording of an individual's
150 movements. Sexually matured virgin beetles of known sex were placed into an individual
151 glass tube (diameter: 5mm, length:65mm) with a flour-disc (40μl of flour/water solution
152 (0.15g/ml) dried overnight) as food inside on one end and sealed with cotton plugs on
153 both ends. The glass tubes were assembled into the DAM5 systems which further were
154 placed into incubators for the recording of activity. The standard rearing conditions were
155 maintained in the incubators throughout the period of recording unless stated
156 otherwise. Before the first day of recording, beetles acclimatized for at least six hours in
157 the activity monitors.

158 **Circadian activity patterns under light entrainment followed by constant darkness (LD**
159 **DD)**

160 For the general characterization of the circadian activity patterns under 12:12
161 Light/Dark (LD) and subsequent constant darkness (DD), we used the Cro1 population
162 (the beetles used belong to the handling control of a knockdown experiment). After sex
163 discrimination we individualized the pupae in wells of a 96-well flat-bottom micro
164 reaction plate (Type F, Sarstedt) containing 0.08 g of flour. 32 adults per sex, which were
165 at least two-week-old post eclosion were used for monitoring locomotor activity for an

166 initial seven days during LD at 30°C (normal rearing temperature) at a light intensity of
167 20 lux followed by seven days of DD.

168 **Circadian activity patterns in constant light after light entrainment (LD LL)**

169 To study the effect of constant light on the circadian activity patterns, we
170 monitored beetles of the Cro1 population in constant light (LL) after entrainment to LD,
171 at two different light intensities: 20 lux and 100 lux. Around two weeks post eclosion,
172 we used 32 adults per sex, which were individualized since sex discrimination at pupal
173 stage, for each light intensity. We recorded their activity for an initial seven days in the
174 LD followed by six days in LL.

175 **Comparison of activity profiles between two laboratory populations**

176 For comparing the circadian activity patterns of the two laboratory populations
177 Cro1 and SB, we separated the pupae according to sex and kept them in groups of 24
178 individuals in petri dishes (Ø10 cm, Waldeck) with *ad libitum* flour. For each population,
179 24 adults per sex which were at least 11 days post eclosion (SB population develops
180 slower than Cro1) were used. The activity recording was carried out in LD (20 lux) for 7
181 days followed by another 7 days of DD. During LD, a technical issue caused the
182 temperature and humidity to drop from 30°C to 25˚C and from 70% to 50%, respectively
183 for 8 hours (from midnight until lights on at 8am).

184 **Circadian activity patterns under temperature entrainment**

185 To test if daily temperature cycles can entrain the locomotor activity patterns in
186 the absence of light, we recorded the beetles' behavior under oscillating temperatures
187 as the zeitgeber. Beetles from the Cro1 population were reared on flour-discs prepared
188 in 96-well plates from day 15 post oviposition onwards. During this time, they were not
189 exposed to light until 21 days post oviposition (as they also served as a control group in
190 a separate experiment, which is not described here**).** Around two weeks post eclosion,
191 beetles were sexed and 24 adults per sex were used for the activity recording for an
192 initial seven days of temperature cycling (12h 25°C/12h 30°C), followed by another
193 seven days of constant 30°C in DD. The activity recording was carried out in complete
194 darkness in both phases to make sure that the temperature change is the only zeitgeber.
195 The experiment was repeated a second time with the same conditions (n=48 per sex
196 during temperature entrainment and n=32 per sex during constant temperature, sample
197 size reduced due to recording error) except that the temperature change was set to
198 ramp for two hours to create a more natural condition of temperature variations.

199 **Activity profiles after knockdown of *Clk***

200 To test, if rhythmic behavior depends on the canonical molecular clock
201 mechanism, we performed RNAi-knockdown (Tomoyasu et al., 2008) of the key
202 circadian clock gene *Clk* at the pupal stage using the Cro1 population.

203 After sex discrimination, pupae were glued with their last abdominal segment
204 onto a glass slide for the dsRNA injection. The pupae were injected between the second
205 and third lowest segment of their abdomen with dsRNA of either *Clk* or jellyfish eGFP ,
206 which served as the control for the dsRNA injection treatment (Schulz et al., 2022). The
207 dsRNA construct of eGFP was produced in our lab (via cloning followed by PCR and *in*
208 *vitro* transcription using the T7 MEGAscript Kit (Ambion by Life TechnologiesTM GmbH,
209 Darmstadt, Gemany)). The dsRNA construct for *Clk* was obtained from EupheriaBiotech
210 (Dresden, Germany) and has previously been used in the ibeetle RNAi screen (Schmitt-
211 Engel et al., 2015, Table S1). A third treatment group served as the pure handling control
212 where the pupae glued on to the glass slides did not receive any injection. dsRNA was
213 injected at a concentration of 1000 ng/µL dissolved in Phosphate-buffered saline (PBS)
214 using a microliter injector (FemtoJet, Eppendorf AG, Hamburg, Germany) and
215 borosilicate glass capillaries (100 mm length, 1.0 mm outside diameter, 0.021 mm wall
216 thickness; Hilgenberg GmbH, Malsfeld, Deutschland). Upon eclosion, the beetles were

217 kept individually in 96-well plates with 0.08 g flour until the start of the activity
218 measurements in the DAM5 system.

219 The activity measurements started two weeks after eclosion. The activity of
220 beetles from all three groups were monitored in LD (light intensity of 100 lux) for seven
221 days followed by seven days in DD.

222 To confirm the successful downregulation following the RNAi treatment, we
223 measured *Clk* expression by qPCR. Fourteen days after eclosion, we shock froze the
224 individuals in liquid nitrogen. Furthermore, we usually pooled 3-4 whole bodies per
225 replicate. All samples were stored at -80°C until the RNA extraction.

226 We extracted total RNA by combining a TRIZOL-chloroform approach with a spin
227 column kit (SV Total RNA Isolation System, Promega (Peuß et al., 2016)). In short, we
228 ground the TRIZOL-submerged samples with a sterile pestle until no larger tissue parts
229 were visible. Samples then went into an ultrasound bath to further ensure disintegration
230 of tissue. After ten minutes of incubation, samples were spun down and the supernatant
231 was mixed with chloroform. Another centrifugation step ensured formation of phase
232 separation. We then transferred the upper aqueous phase mixed with lysis buffer onto
233 the spin column and proceeded according to manufacturer's protocol. Finally, RNA was
234 eluted with 80µl nuclease free water and stored at -80°C until further use.

235 For the qPCR, we first reverse transcribed RNA to cDNA using the RevertAid First
236 Strand cDNA Synthesis Kit (Thermo Scientific) and the provided oligo$(dT)_{18}$ primer,
237 following the manufacturer's instructions. Before the cDNA was used in the qPCR it was
238 diluted 1:5 with nuclease free water.

239 We performed qPCRs with technical duplicates and two housekeeping genes
240 *rp49 and RpL13 a* to normalize target gene expression. Amplification efficiency of
241 primers was previously established (Table S2) as well as the uniform expression of the
242 housekeeping genes across sexes and treatments. The qPCR ran on a LightCycler 480
243 System (Roche) with KAPA SYBR Fast mastermix (Sigma-Aldrich).

244 For each RNAi treatment and control, we measured gene expression separately
245 for males and females in four biological replicates. We included samples in the
246 downstream analysis if the standard deviation between the technical replicates
247 remained below 0.5 and the melting curves showed only one clear peak.

248 **Behavior analyses**

249 After two weeks of recording, we extracted the raw data of locomotor activity in
250 activity bins of 30 minutes using DAMFileScan software (TriKinetics). We looked for
251 activity patterns from the average histograms during different entrainment and

252 constant conditions, and differences in average activity levels, and proportion of
253 rhythmic beetles using data from individual beetles.

254 For the average comparison of diel patterns in movement, average histograms
255 of mean activity in 30-minute intervals for all individuals belonging to the same
256 treatment and sex group across the days of the same environmental condition, were
257 generated using the Fly-toolbox (Levine et al., 2002b).

258 Individual beetles were scored to be rhythmic or arrhythmic based on a
259 combination of qualitative and quantitative measures using double plotted actograms,
260 autocorrelation (Levine et al., 2002b) and χ^2-periodograms (Sokolove and Bushell, 1978)
261 generated using Fly-toolbox in MATLAB (MATLAB and Statistics Toolbox Release 2021b).
262 Double plotted actograms were used for the visual analysis to identify rhythmic
263 patterns. Each row in a double plotted actogram displays the activity in two consecutive
264 days for which the activity of the second day is replotted in the left part of the next row
265 to inspect for the activity patterns horizontally and vertically. A beetle is categorized as
266 rhythmic if activity patterns are repeated for at least half of the days of activity
267 recording. To reduce the subjectivity from the visual detection of rhythmicity,
268 quantitative thresholds from autocorrelation and periodograms were also used in
269 combination with the visual inspection of actograms (Levine et al., 2002b).

Autocorrelation graphs identify patterns in the data by examining the correlation between the movement signal and a time-shifted version of itself. This analysis helps detect both the strength of the rhythm (RS-value) and the duration of the identified period (p). The χ2-periodogram assesses rhythmicity by testing various potential periods within the data and evaluating their fit, represented by the Qp value. The dominant signal period is determined based on the highest Qp value, while a 95% confidence interval is used to evaluate the significance of the chosen period. Additionally, the h-value indicates to which extend the Qp value of the selected period exceeds this confidence interval. A beetle was categorized as rhythmic only if it met all three criteria: having a clear rhythmic pattern in the actogram, RS>1 in the autocorrelation and h > 10 in the periodogram.

The circular phase plots showing the phase of individual beetles (Levine et al., 2002b) were also plotted using MATLAB. For the analysis of activity levels, activity in 1-minute intervals were averaged over all monitoring days of a specific condition for individual beetles. The bar graphs, plotting proportion of rhythmic beetles and boxplots for the average activity were generated using ggplot2 (Wickham, 2016) in RStudio version 2021.09.1 (RStudio Team, 2021) under R version 4.1.2 (R Core Team, 2021). We did some final editing of supplementary figure panels using Inkscape (Developers, Inkscape Website, 2020)

289 **Statistics**

290 All the statistical analysis for the rhythmicity and average activity levels were
291 performed using R Studio employing additional package glmmTMB (Brooks et al., 2017).

292 The analysis for rhythmicity was done on binary data indicating either presence
293 or absence of consistent rhythmic patterns for individual beetles. The analysis for the
294 activity levels was done on the activity in 1-min bin averaged over all the days of a
295 condition for individual beetles. The differences in the proportion of rhythmic beetles
296 and activity levels were analyzed in generalized linear mixed effect models (GLMMs)
297 (Table S3) with the according error distributions. The final models were derived after p-
298 value based stepwise model simplification.

299 For the statistical analysis of gene expression after RNAi treatment, we used the
300 Relative Expression Software Tool (REST2009, Pfaffl et al., 2002). The tool is designed to
301 compare the gene expression between two groups – in our case, expression in the
302 knockdown treatment was compared to the expression in the RNAi control group. REST
303 employs a mathematical model, which takes the PCR efficiencies of the primers into
304 account when calculating expression ratios from the mean crossing point deviation. It
305 then performs a randomization test to determine statistically significant differences
306 between the treatment groups.

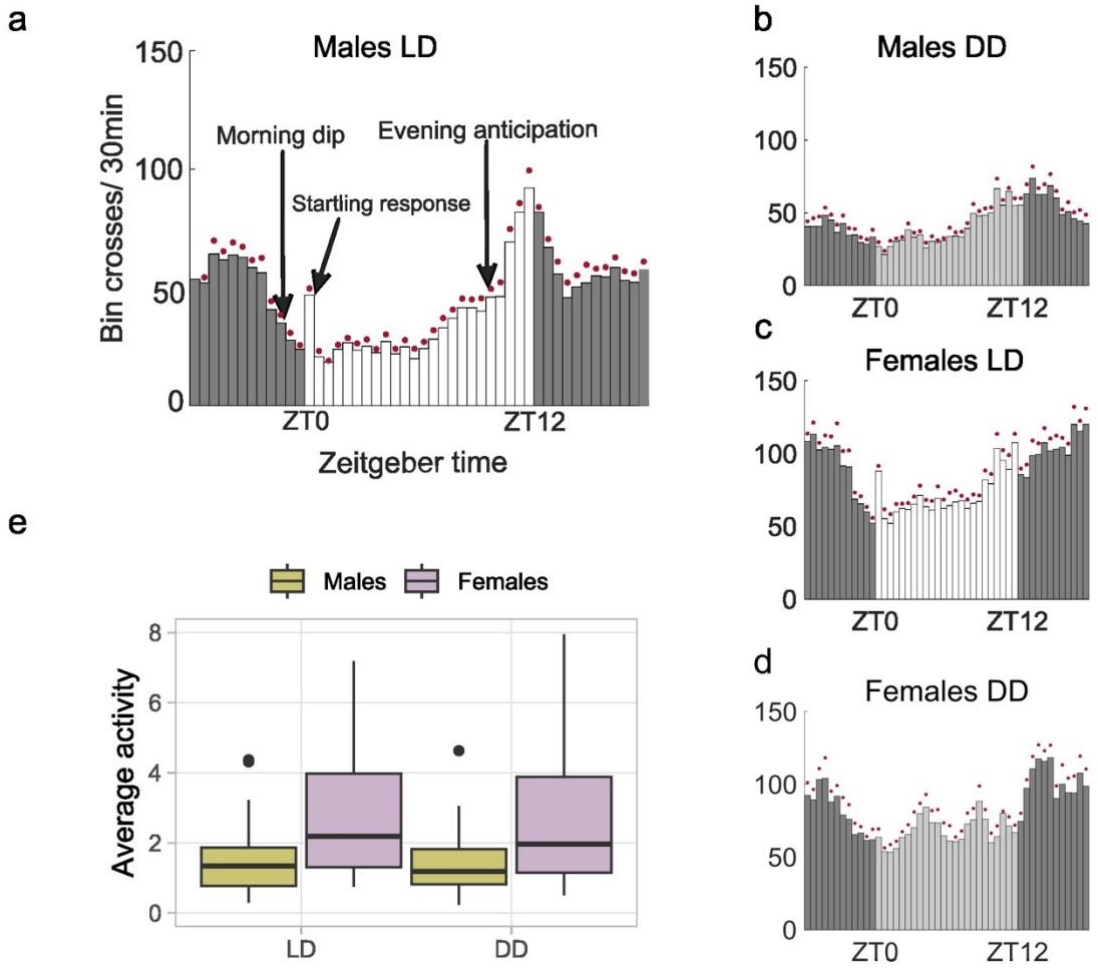
307 **Results**

308 To foster our understanding of the workings of the molecular clock in flour
309 beetles and to further characterize the circadian patterns in their behavior, we first
310 monitored and analysed the locomotor activity rhythms of an outbred population (Cro1)
311 of *T. castaneum.* We tested the beetles' behavior under different light and temperature
312 cycles for both sexes. Additionally, we compared the outbred strain (Cro1) to an inbred
313 strain (SB), which has adapted to laboratory conditions for decades, to infer strain
314 specific behavioral differences. Finally, we downregulated *Clk* gene expression via RNAi
315 to investigate the role of this key circadian clock gene in generating the behavioral
316 patterns.

317 **Locomotor activity patterns under light entrainment**

318 We monitored the locomotor activity patterns of beetles from the Cro1
319 population under LD (20 lux) conditions followed by DD. The average activity profiles of
320 beetles showed a clear and repeatable diurnal rhythm (Fig 1 a-d). The daily average
321 activity patterns were characterized by a consistent reduction in activity levels just
322 before the onset of morning light in all the experiments, which we refer to as 'morning
323 dip' (Figure 1 a). They reacted with a startle response of increased activity observed in
324 response to the onset of the morning light stimulus (Figure 1a). This response fades

325 quickly within 30 minutes and is not observed under DD conditions. Upon more natural
326 like conditions with a linear ramping**,** extending the lights-on transition to 2 hours, the
327 startle response either disappeared or appeared at the end of the ramping period, but
328 as a lower peak compared to the non-ramping conditions (Fig S2). Further, beetles
329 gradually increased their activity levels throughout the late afternoon, a behavior which
330 we accordingly (and in analogy to a similar pattern observed in *D. melanogaster*
331 (Hamblen-Coyle et al., 1992)) call evening anticipation (Figure 1a). With the start of the
332 dark phase in LD, the beetles slightly reduced their activity levels from their highest peak
333 during the evening anticipation but remained highly active throughout the night until
334 they reduced their activity considerably during the morning dip (Figure 1 a and c).



335

336 **Figure 1: Locomotor activity patterns of *T. castaneum*.** a-d: Average histograms
337 showing mean activity of Cro1 beetles over the 7 days of recording in 30-minute bins
338 under LD (light entrainment of 20 lux) and DD; e: Boxplots showing activity in 1-minute
339 bin for individual beetles averaged over 7 days in LD and DD (males: n = 31, females:
340 n=32).

341 **Locomotor activity patterns under constant darkness**

342 In DD, the beetles' average activity profiles mirrored those observed during the
343 LD except for the obvious absence of the startle response. Before the start of the
344 subjective morning, there was a clear reduction of activity similar to the morning dip
345 and a gradual increase of activity throughout the subjective late afternoon that
346 resembled the evening anticipation (Fig 1 b and d). In addition to the generally higher
347 levels of activity observed in females compared to males, the average histograms also
348 illustrate the irregular and noisy patterns exhibited by the females (Fig 1 a-d). The
349 analysis of individual average activity in 1-minute bins also reveals a significantly lower
350 average activity for males (GLMM, estimate = -0.588, $p < 0.001$, Fig 1e). In terms of
351 individual rhythmicity, 52% of males and 22% of females showed rhythmic patterns in
352 LD whereas the proportion of rhythmic beetles was similar (32% for males and 28% for
353 females) in DD conditions (Table 1). However, upon statistical analysis of individual
354 rhythmicity, there was no significant effect for the sex (GLMM, estimate = 0.338 $p =$
355 0.665) or the light condition (GLMM, estimate = -0.491 $p=0.489$) whereas there was a
356 weak trend for the interaction between the effects of sex and light condition (GLMM,
357 estimate = 1.727, $p = 0.089$)

358 **Variations in locomotor activity patterns**

359 A substantial amount of individual variation can be seen in the rhythmic patterns
360 in LD (Figure 2 a-f). Among the rhythmic beetles, not all show identical patterns. Activity
361 peaks of many beetles start just before the lights turn off, and among these, some have
362 their peak of activity for a short duration before the lights turn off (Fig 2 c) or extending
363 further throughout the dark phase of the LD (Fig 2 a). The individual variations in phase
364 of their activity can also be seen from the circular phase plot (Fig 2 g). The females have
365 more coherent phase compared to the males. There was variation in terms of whether
366 the beetles could consistently maintain the rhythmicity throughout both light conditions
367 as well (Fig 3 a-d). Not all beetles that were rhythmic in LD were able to maintain their
368 rhythmicity during DD. 9 out of 31 males and 2 out of 32 females were rhythmic in LD
369 but showed arrhythmic behavior in DD. There was a small proportion of beetles (3 out
370 of 31 for males and 4 out of 32 for females) that showed rhythmic behavior only in DD
371 (Fig 3c, e).

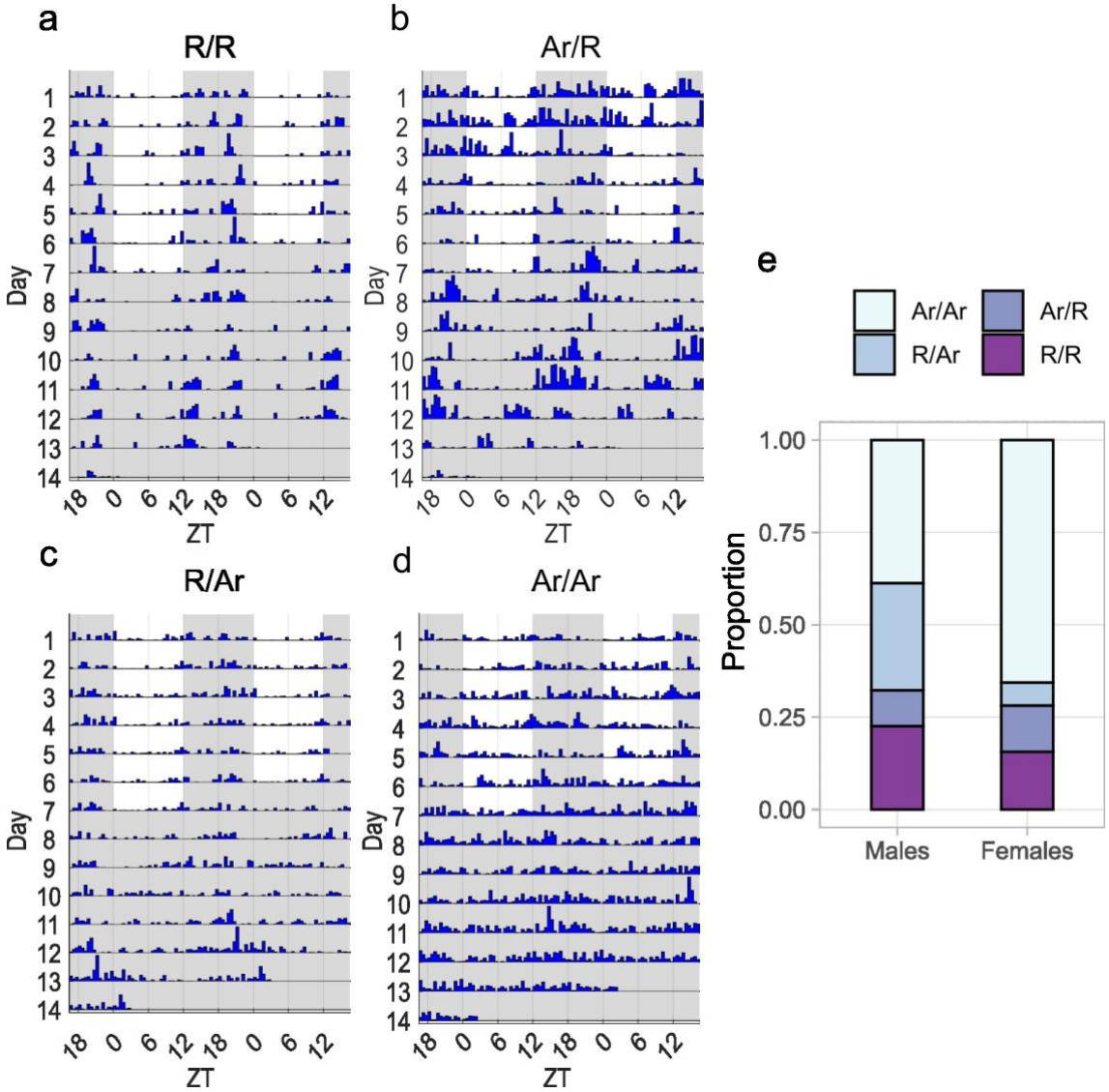


Figure 2: Variation among individuals. a-f Variation in behavioral patterns among individuals during LD; g: circular phase plot showing phases of beetles in LD based on data from the first 3 days. The lengths of the line from the centre represents the coherence of phases within a group.

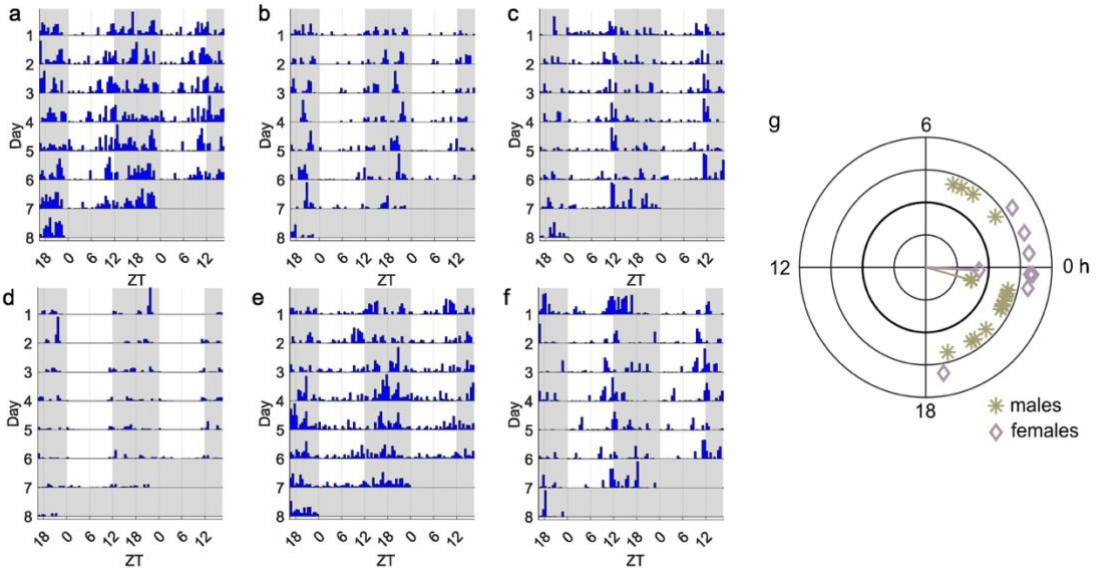


Figure 3: Maintenance of rhythmicity under LD and DD. a- d: Individual actograms showing different categories of beetles according to their status of being rhythmic (R) or arrhythmic (Ar) under the two conditions of LD (first 7 days) and DD (last 7 days) (R/R - rhythmic in LD and DD, R/Ar – rhythmic in LD but not in DD, Ar/R – Rhythmic in DD but not in LD, Ar/Ar – Arrhythmic in both LD and DD), e: stacked bar plot showing the proportion of these categories separately for males and females.

Locomotor activity patterns under constant light conditions

When LD was followed by constant light conditions (LL), the average activity profiles of beetles resembled those observed in DD, however, overall rhythmicity compared to DD was reduced under both light intensities tested (20 lux and 100 lux; Fig 4 and Fig S3, respectively, Table 1). When examining individual rhythmicity, males

showed a significantly higher proportion of rhythmic individuals compared to females (GLMM, estimate = 1.711, $p < 0.001$, Fig 4e). Furthermore, the proportion of rhythmic beetles experienced a significant decrease under constant light conditions compared to LD (GLMM, estimate = -3.193 $p < 0.001$, Fig 4e). There was an overall significant effect of light intensity on the rhythmicity (GLMM, estimate = -1.002, $p = 0.03$, Fig 4e). There was a significant interaction between light intensity and the light condition, with a sharper drop in rhythmicity from LD to LL for the higher light intensity (GLMM, estimate = 1.772, $p = 0.026$, Fig 4e).

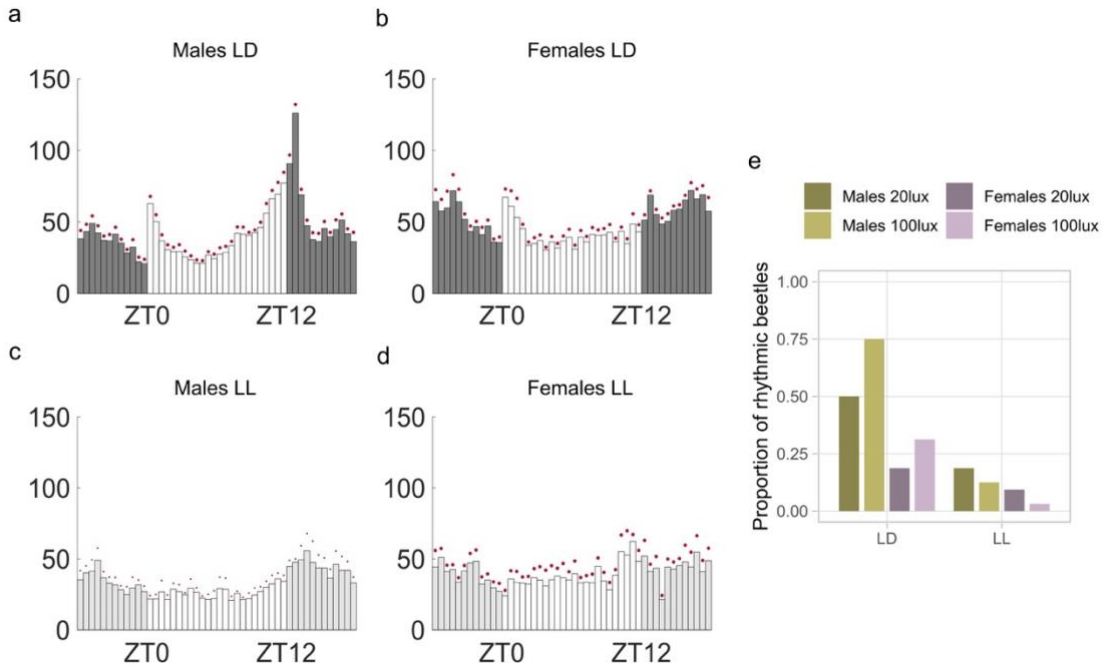


Figure 4: Rhythmicity under LL conditions. a-d Average histograms showing mean activity for all the beetles over the 7 days of recording under LD (light entrainment of 20 lux) and LL (20 lux) (Average histograms for 100 lux given in Fig S3), e: Proportion of rhythmic beetles in LD and LL for two light intensities;20 lux and 100 lux (males: n = 32 per light intensity, females: n=32 per light intensity)

Population differences in locomotor activity and behavioral patterns

Both Cro1 and SB populations had similar behavioral patterns (Fig 5 a-h), but the average activity was significantly lower for the SB population during LD (GLMM, estimate = -0.667, $p < 0.001$, Fig 5 j) and DD (GLMM, estimate = -0.368, $p = 0.04$, Fig 5k). The

average activity of females was significantly higher than that of males in LD (GLMM, estimate = -0.624, $p < 0.001$, Fig 5 j) but not in DD (GLMM, estimate = -0.118, $p = 0.512$, Fig 5k). While examining individual rhythmicity, the SB population showed a significantly higher proportion of rhythmic beetles in LD (GLMM, estimate = 1.116, $p = 0.006$, Fig 5i). The effect of light condition was also significant with a higher proportion of beetles observed in LD (GLMM, estimate = 1.761, $p < 0.001$, Fig 5 i). However, there was no significant effect of sex on rhythmicity (GLMM, estimate = 0.413, $p = 0.279$, Fig 5 i).

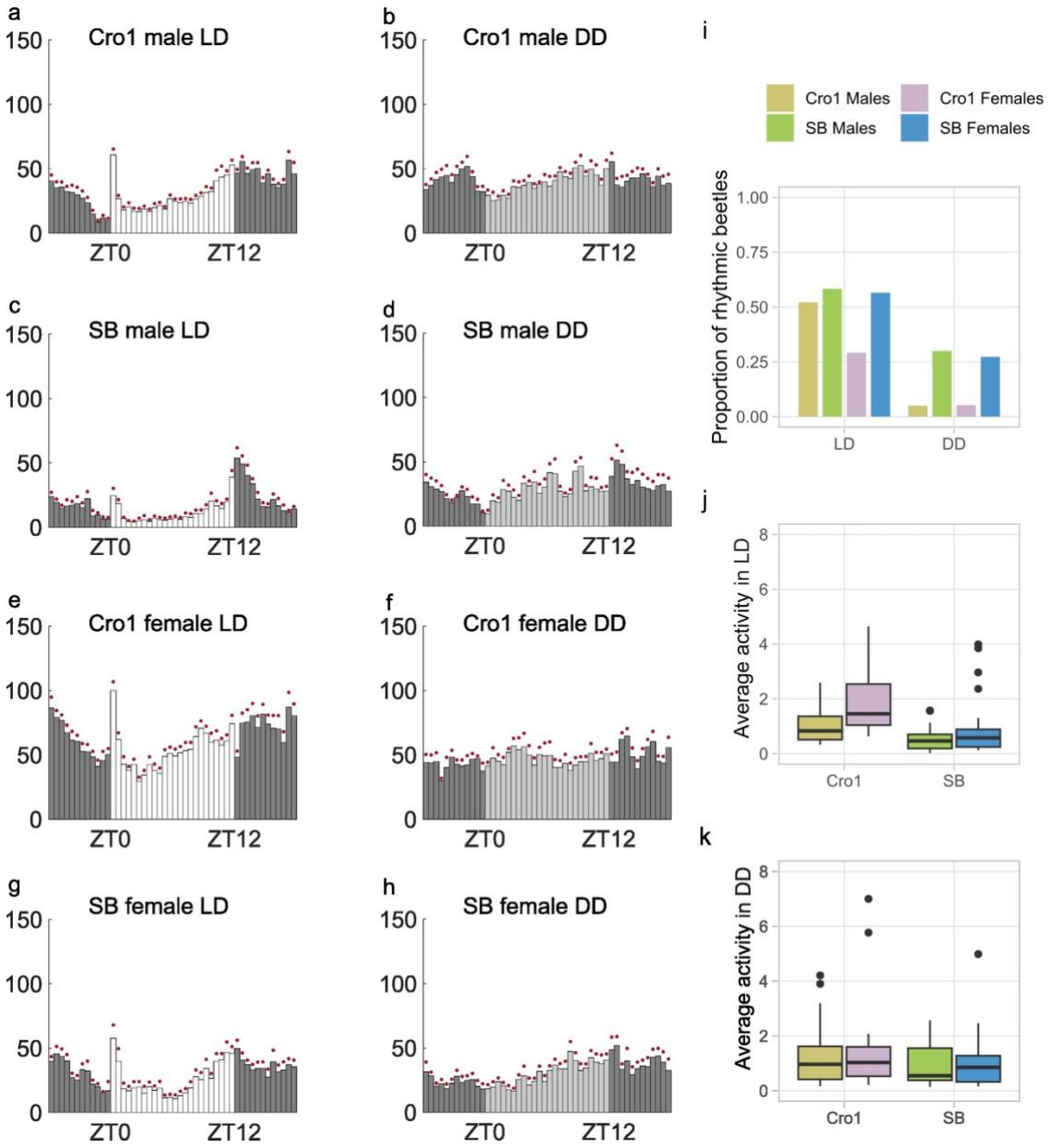


Figure5: Population differences in rhythmicity. a-h: Average histograms showing mean activity for all the beetles over the 7 days of recording under LD (light entrainment of 20 lux) and DD for Cro1 and SB populations, i: Proportion of rhythmic beetles in LD (Cro1

418 males: n = 23 , Cro1 females: n=24, SB males: n=24, SB females: n=23) and DD (Cro1
419 males: n = 20 , Cro1 females: n=19, SB males: n=20, SB females: n=22) j and k: Boxplots
420 showing activity in 1-minute bin for individual beetles averaged over 7 days in LD (j,
421 Cro1males: n = 23, Cro1 females: n=24, SB males: n = 24, SB females: n = 23) and DD (k,
422 Cro1 males: n = 22, Cro1 females: n=20, SB males: n = 21, SB females: n = 22)

423 **Locomotor activity patterns during and after temperature entrainment**

424 Similar to the behavior in LD, the beetles showed activity rhythms in a 12 hr: 12
425 hr temperature cycle of 25^0C: 30^0C in constant darkness (DD) (Fig 6 a-d, Fig S4). However
426 rhythmic behavioral patterns, apart from the prominent peaks of activity at the start of
427 temperature change in the subjective morning and evening, were less prominent
428 compared to LD. The peaks of activity at the temperature transitions resembled the
429 startle response observed after lights-on in LD and were not retained in constant
430 temperature conditions (Fig 6 a-d).

431 After the temperature cycles, only males showed a clear rhythmic activity
432 pattern under constant temperature conditions (Fig 6 c). The proportion of rhythmic
433 beetles significantly differed between sexes (GLMM, estimate = 1.418, p = 0.018, Fig 6
434 e).

435 Upon the more natural-like temperature cycling in constant darkness with a
436 ramping of temperature change within 2 hours, the activity patterns in the entrainment

437 phase appeared stronger compared to the entrainment with a square-wave of
438 temperature cycling (Fig S5). Both the startling peaks disappeared and there was a clear
439 dip in the morning activity as well as a stronger evening anticipation in the entrainment
440 phase resembling the pattern in LD (Fig S5). In the subsequent constant temperature
441 conditions, both males and females retained these activity patterns. There was no
442 difference in the proportion of rhythmic beetles between the sexes (GLMM, estimate =
443 0.185, p = 0.635, Fig S4 E) as well as between temperature cycles and constant
444 temperature conditions (GLMM, estimate = -0.427, p = 0.283, Fig S4 e).

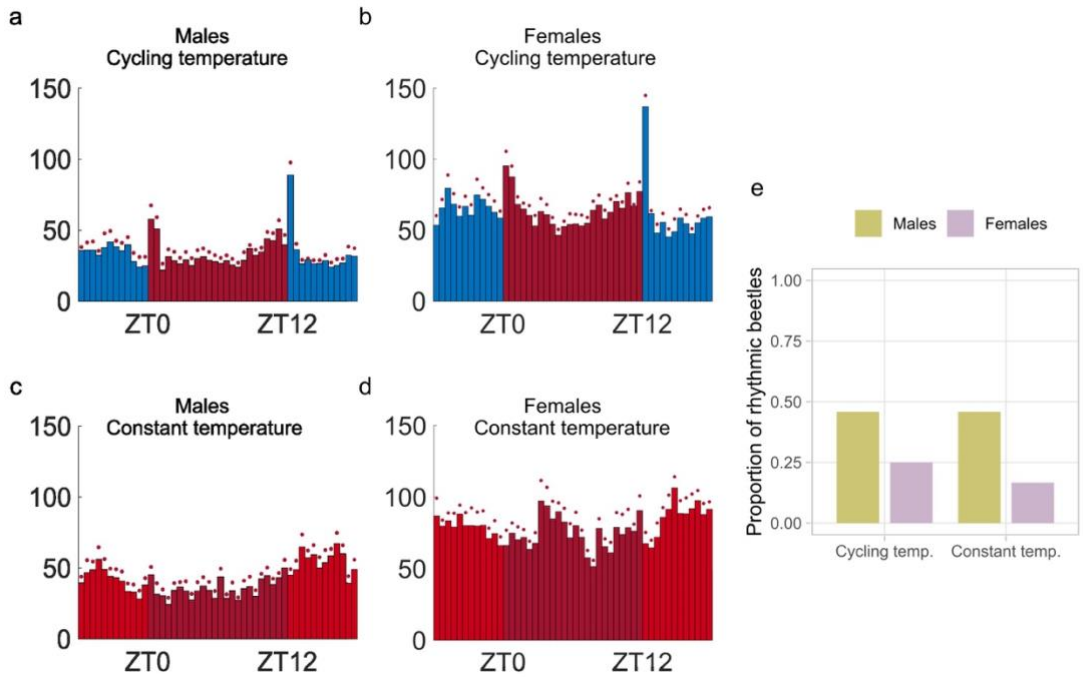
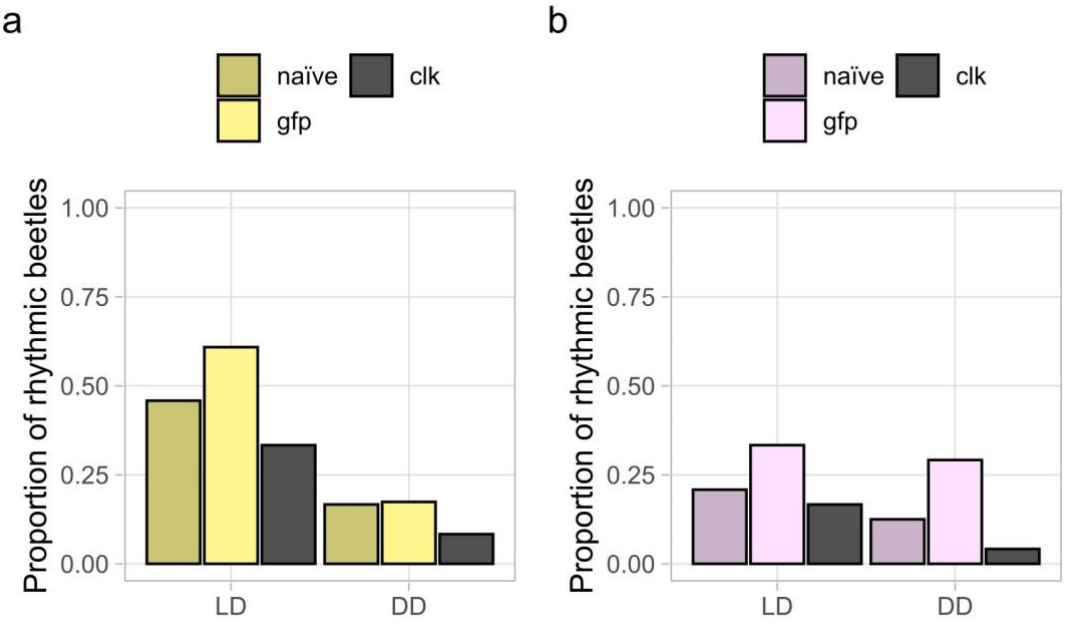


Figure 6: Temperature entrainment. a-d Average histograms showing mean activity for all the beetles over the 7 days of recording under temperature cycling and subsequent constant temperature, both under constant darkness, e: Proportion of rhythmic beetles in cycling temperature and constant temperature (males: n = 24 per temperature condition, females: n=24 per temperature condition)

Locomotor activity patterns upon knockdown of *Clk*

We downregulated the key circadian clock gene *Clk* using RNAi in the pupal stage to study the subsequent changes in activity profiles.

454 *Clk* gene expression was significantly different between the sexes (Table 2) in the
455 RNAi control. There was a significant reduction in *Clk* gene expression compared to the
456 RNAi control, indicating that dsRNA-mediated knockdown was efficient (Table 3).
457 Following the *Clk* knockdown, there was also a significant reduction in rhythmicity
458 (GLMM, estimate = -1.369, p = 0.005) in comparison to the RNAi control beetles,
459 showing that *Clk* function is important for behavioral rhythmicity (Fig 7). There was a
460 significant interaction effect of sex and light condition as well on the rhythmicity (GLMM,
461 estimate = 1.391, p = 0.04) (Fig 7).



462

463 **Figure 7: Rhythmicity upon knockdown of *Clk*.** Proportion of rhythmic individuals in
464 adults following the knockdown treatment at pupal stage for *Clk* in males (a) and females

465 (b) (LD: n =24 per sex and treatment groups, DD: n=24 per sex and treatment groups

466 except for RNAi control males (n = 23))

467 **Table 1: Proportion of rhythmic beetles in different experiments**

Experiment		**Percentage of rhythmic beetles**			
		Entrainment		**Constant condition**	
		Males	**Females**	**Males**	**Females**
LD (20 lux)/DD		51.6%	21.9%	32.3%	28.1%
LD LL	20 lux	50.0%	18.8%	18.8%	9.4%
	100 lux	75.0%	31.3%	12.5%	3.1%
Population difference in LD DD	Cro 1	52.2%	29.2%	5.0%	5.3%
	SB	58.3%	56.5%	30.0%	27.3%
Temperature entrainment		45.8%	25.0%	45.8%	16.7%

Temperature entrainment with ramping		35.4%	33.3%	28.1%	21.9%
Knockdown of *Clk*	Naïve	45.8%	20.8%	16.7%	12.5%
	RNAi control	58.3%	33.3%	17.4%	29.2%
	Clk knockdown	33.3%	16.7%	8.3%	4.2%

468

469 **Table 2: Sex difference in gene expression of *Clk***

Relative gene expression of males compared to females	**95% C.I.**	**n**	**p value**
0.312	0.158 - 0.795 ctrl vs ctrl (male vs male): 0.381 - 2.642	4	**0.02**

470

471

472 **Table 3: Knockdown of *Clk***

Sex	Relative expression compared to RNAi control	95% C.I.	n	p value
Male	0.463	0.227 - 1.273 ctrl vs ctrl: 0.381 - 2.642	4	**0.031**
Female	0.531	0.279 - 0.988 ctrl vs ctrl: 0.539 - 1.858	4	**0.028**

473

474 **Discussion**

475 We used the Drosophila Activity Monitoring system (DAM) to perform a detailed
476 characterization of the average as well as individual locomotor activity profiles of adult
477 *T. castaneum* extending its application to study behavioral patterns in another
478 important model organism. We recorded the activity of lab-adapted populations with
479 high temporal resolution and described the activity patterns under light entrainment,
480 constant dark conditions and for the first time to our knowledge, under thermal
481 oscillations and constant light conditions. The beetles anticipated environmental
482 transitions and even though less robust, maintained their rhythmicity in DD and LL. We
483 showed that a substantial amount of individual variation exists in the diel patterns of

484 this cryptozoic species. The knockdown of *Clk* significantly reduced rhythmicity, strongly
485 supporting the existence of circadian clock in *T. castaneum* and its control of behaviors.
486 The finding that temperature cycling acts as a zeitgeber for the beetle clock, in
487 addition to light, is consistent with our expectations, considering that beetles are
488 exposed to cycling temperatures in their natural habitats of grain silos (Ziegler et al,
489 2021) as well as in their supposed original habitats under tree bark (Good, 1936;
490 Sokoloff, 1974). Studies in many other insects including fruit flies (Glaser and Stanewsky,
491 2005) and honeybees (Giannoni-Guzmán et al., 2021) have documented the role of light-
492 dark cycles as well as temperature oscillations as zeitgebers. The notable patterns of the
493 average activity profiles under both zeitgebers included a consistent drop of activity just
494 before the change of conditions in the morning which we refer to as 'morning dip' and
495 the gradual increase of activity throughout the late afternoon known as evening
496 anticipation. These patterns emerged even before the zeitgeber conditions change as
497 well as appeared consistently in constant conditions, indicating an anticipatory response
498 to the predicted changes resulting from their entrained molecular clock. Other insects
499 such as *D. melanogaster* (Dubowy and Sehgal, 2017) and *Plutella xylostella* (D. Wang et
500 al., 2021) also show such gradual increase in activity anticipating the transitions of
501 environmental conditions. However, a gradual decrease of activity (morning dip)
502 towards the time of dark-light transition is rather atypical.

503 The beetles reacted with a startle response of an exceptionally heightened
504 activity to the light turning on in the morning as well as to the change of temperature in
505 the subjective morning and evening. The absence of these startle responses in constant
506 conditions as well as their disappearance or strong reduction under more natural-like
507 conditions of ramping temperature or light conditions confirms that they are not
508 mediated by the circadian clock but are instead reactions to sudden changes in
509 environmental conditions. Similar startle responses immediately following the dark-light
510 transition were observed in *Ae. aegypti*, and, consistent with our study, these responses
511 were not present in constant darkness (Gentile et al., 2009) .

512 Average histograms consistently exhibited evening anticipation in constant
513 conditions as well as following the entrainment phase for both light and temperature as
514 zeitgebers. A steep evening anticipation was characteristic of the average activity
515 profiles under light entrainment, especially for males. The evening anticipation aligns
516 well with the time of day when mating rates of the *T.castaneum* reach their maximum
517 (Z. Wang et al., 2023). In red flour beetles, males initiate the copulatory behavior and
518 must mate multiple times to ensure fertilization success, especially because of the last
519 male sperm precedence (Fedina and Lewis, 2008). An increased activity of males in
520 search of mates could be a potential reason why males possess a strong and clear
521 evening anticipation peak compared to the females. Similarly, in *P. xylostella*, males

522 were shown to have better rhythmic features than females under constant darkness in
523 low temperature conditions (D. Wang et al., 2021). Such sex-specific differences in
524 activity profiles are commonly observed in many insect species and are often associated
525 with their mating behavior. For example, in *Anopheles gambiae*, males have an earlier
526 flight activity peak compared to females, consistent with the fact that males aggregate
527 to form swarms to which females subsequently enter (Rund et al., 2012). In addition to
528 the difference in evening anticipation, the overall activity levels of females were
529 significantly higher than those of males, as was also reported recently (Benita et al.,
530 2024). we also observe a large inter-individual variation in phase for the males
531 compared to females. Additionally, males generally exhibit a higher proportion of
532 rhythmicity, which may be attributed again to the necessity for males to initiate
533 copulation behavior, thereby requiring precise timing of such behaviors.

534 In addition to the individual variation in phase, there was substantial variation in
535 the individual activity patterns including differences in the duration of peak activity,
536 levels of activity at different times, and the ability to maintain rhythmic behavior under
537 constant conditions following entrainment. A study in the cave beetle species,
538 *Laemostenus latialis* also showed similar variability in individual behavioral patterns
539 (Pasquali and Sbordoni, 2014). Another study observed significant diversities in
540 circadian behaviors in social insect species compared to the solitary species like fruit flies

541 (Giannoni-Guzmán et al., 2014). Since *T.castaneum* is a non-social yet group living insect,
542 one could speculate that the dual role of the pheromone, 4,8-dimethyldecanal (DMD)as
543 an aggregation as well as a sex attractant for females (Levinson and Levinson, 1995)
544 enables individuals to rely on their conspecifics for timing their own mating rather than
545 a strict reliance on their circadian clock. Another possibility is that variability in their
546 peak activity timings could help in avoiding any possible male-male competition.

547 The proportion of rhythmic beetles considerably decreased in DD conditions
548 especially for males **.** The presence of all clock gene homologs known in *Drosophila*
549 (Richards et al., 2008) indicates that *T. castaneum* possesses a functional circadian clock.
550 Therefore, the decrease in rhythmicity in constant darkness compared to that under
551 light entrainment could possibly indicate a weak link or uncoupling between the
552 endogenous clock and the locomotor behavior under constant darkness (Abhilash et al.,
553 2017) as seen in the case of blind mole rats (Beale et al., 2016) and in *Drosophila* species
554 inhabiting higher latitudes with weakly rhythmic environments (Bertolini et al., 2019).
555 The inter-individual variability seen in locomotor activity profiles could also possibly be
556 the result of a weak behavioral regulation by the endogenous clock (Beale et al., 2016).
557 One could speculate that such a weak coupling or even uncoupling might be due to the
558 absence or reduced selective pressure for circadian functions in their possible ancestral
559 natural dark habitats such as rotting logs and tree barks as well as in their recent, also

560 largely dark habitats of grain silos (Good 1936; Sokoloff 1974). The possible chance of
561 relying on olfactory cues from conspecifics for mating initiation might have also resulted
562 in a weak selection pressure for the evolution of a strong endogenous clock or a strong
563 coupling of the clock with behaviors.

564 There was a significant decrease in the proportion of rhythmic beetles in
565 constant light conditions, more pronounced under higher light intensity, even if weak
566 rhythmic patterns were observable in the average histograms. In wild-type D.
567 *melanogaster*, strong constant light leads to arrhythmic behavior (Konopka et al., 1989,
568 Matsumoto et al., 1994) because of the light-dependent TIM degradation by CRY1
569 which conveys photic signals to the circadian clock (Ceriani et al., 1999). The beetles are
570 not expected to have such an arrhythmic behavior as they possess only the light-
571 insensitive cryptochrome (CRY 2) (Yuan et al., 2007). However, most likely retinal and/or
572 potentially also unknown extraretinal photoreceptors play a role (Helfrich-Förster,
573 2020) in photic entrainment, which could then possibly affect the rhythmicity under
574 such constant light conditions. A similar reduction of rhythmicity under constant light
575 conditions was reported for *Nasonia vitripennis*, which also possesses only the light-
576 insensitive CRY 2 (Bertossa et al., 2014), whereas some other closely related *Nasonia*
577 species retained their rhythmicity (Bertossa et al., 2013). A study in linden bug, which
578 also possesses only CRY 2, revealed a deteriorating effect of constant light on

579 rhythmicity even though around 40% of individuals showed strong rhythmicity under
580 such constant light conditions (Kaniewska et al.,, 2020)

581 To test if the observed behavioral rhythms depend on a molecular circadian
582 clock, we attempted the knockdown of the key circadian clock gene *Clk* in the pupal
583 stage and subsequently monitored locomotor activity profiles at the adult stage. Indeed,
584 rhythmicity in *Clk* dsRNAi animals was significantly reduced compared to the control *GFP*
585 dsRNAi beetles, strongly supporting a role for a canonical CLK-dependent
586 transcriptional/translational circadian feedback loop in *T. castaneum*. In the future,
587 double knockdowns of different clock genes could be performed to induce more severe
588 clock disruption and phenotypes.

589 Since *T.castaneum* is a non-social but gregarious species, further experiments to
590 study their circadian rhythms in a group setting would be the next step. The social
591 influence on circadian rhythms has not only been demonstrated in social insects, but
592 also for a solitary species like *Drosophila* for which this is most likely mediated by
593 olfactory cues (Levine et al., 2002a). Studies of circadian rhythms of *T.castaneum* in a
594 social setting are especially important, because it is already known that an exposure to
595 the male aggregation pheromone increases the expression of the *timeless* clock gene in
596 these beetles (Rath et al., 2021).

597 As *T. castaneum* is a ubiquitous pest, knowledge about its diel patterns in
598 behavior under different external environmental cues can be useful in pest management
599 strategies, for instance in light traps. Recently, circadian clock research is gaining more
600 importance in immune system homeostasis (Haspel et al., 2020). Improved knowledge
601 about the circadian clock and the rhythms it controls in *T. castaneum,* which is an
602 excellent model to study insect immunity (Altincicek et al., 2008; Ferro et al., 2019), can
603 be beneficial for the emerging research in the intersection of circadian function and
604 immune regulation which has potential clinical relevance. It is remarkable that despite
605 the absence of strong light cues in their original habitats, the molecular clock of flour
606 beetles still shows a strong entrainment to light as well as that many beetles can retain
607 this rhythmicity under constant conditions. The considerable variation seen in different
608 aspects of the diel patterns along with its status as a group living yet non-social insect,
609 renders this organism a unique model for investigating circadian clocks and the different
610 behavioral and physiological parameters they control. This complements studies in
611 other insect groups, contributing to a comprehensive understanding of circadian
612 rhythms across different insect species.

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Conflict of interest statement

The authors have no potential conflicts of interest.

Note

Supplementary material for this article including the final data files is available online.

Author contributions

JK and RS conceived the ideas. JK, RS, MO, RR, NS and TP planned the experimental procedure. RR, TP, NS and PM carried out the experiments and RR carried out the statistical analyses together with NS, MO, TP and PM. RR wrote the first draft which was revised by NS and TP. RR and TP made the figure panels. All authors contributed to writing of the article in further revisions and approved the submitted version.

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