Donor plasmid generation for the mutagenesis of *timeless* in *Drosophila suzukii* using CRISPR/Cas9

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Abstract

Drosophila suzukii (spotted wing fruit fly) is an invasive pest species that lays its eggs inside ripening fruits, causing significant crop losses. Its global range has expanded drastically in recent years and the species now occupies a wide variety of environments. Animals rely on their circadian clock, an endogenous timekeeping system, to anticipate daily changes in the environment (e.g. day-night cycles) and adjust their behaviour and physiology accordingly. *D. suzukii* is closely related to the circadian clock model *D. melanogaster,* which makes genetic manipulations of the species easier. It shows strong seasonal adaptations, making it an ideal species to investigate how the circadian clock interprets photoperiodic information to prepare for seasonal changes.

This project aims to create transgenic *D. suzukii* flies in which *timeless*, an essential clock gene is disrupted. Specifically, we focused on generating a donor plasmid that can be used for homology directed repair-mediated transgenesis by the CRISPR-Cas9 system. Additionally, the daily behaviour of wild type *D. suzukii* flies alone and in pairs was measured in locomotor experiments. This dataset can help assess the effects of *timeless* disruption once mutant flies are available.

Introduction

The circadian clock

Most organisms on Earth are exposed to the daily cycles of light and darkness. Anticipating these periodic changes poses a significant fitness advantage, so all higher organisms have evolved complex internal clocks (Jarabo and Martin, 2017). The circadian clock naturally operates in around 24 hours long cycles, adapting our behaviour and physiology to the time of the day. It maintains its rhythmicity even when isolated from all environmental cues, demonstrating its endogenous nature. The clock can be synchronised to external cues called Zeitgebers, such as light, temperature and social signals (Peschel and Helfrich-Förster, 2011). An essential function of the circadian clock is the modulation of locomotor activity, which allows animals to occupy distinct temporal niches (Peschel and Helfrich-Förster, 2011).

The fruit fly *D. melanogaster* has proved to be an ideal model to study the circadian clock. Its typical crepuscular activity pattern, meaning that its most active during dusk and dawn, is closely regulated by the clock and easy to observe in the laboratory. The simplicity of the fly brain and the diverse genetic tools available enabled the successful unravelling of molecular and neural events behind the clock (Peschel and Helfrich-Förster, 2011). A group of ~150 neurons per hemisphere in the brain forms the basis of the fly master clock (Jarabo and Martin, 2017). The level of clock proteins in these cells cycles throughout the day, resulting from the well-regulated interaction of various genes (Figure 1). *period* (*per*) and *timeless* (*tim*) are essential in this process. They regulate their own expression in an elaborate

negative feedback loop, with the help of clock (CLK) and cycle (CYC) transcription factors. The oscillation in the levels of these clock proteins affects diverse cellular functions, leading to circadian changes in physiology and behaviour (Peschel and Helfrich-Förster, 2011).

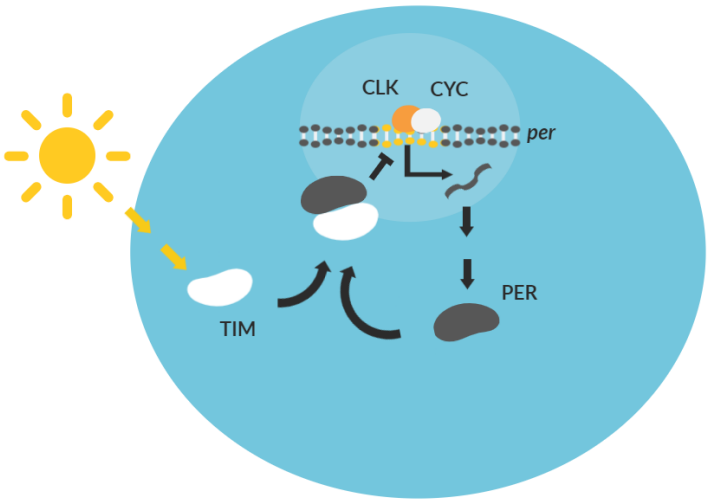

Figure 1: The molecular basis of the D. melanogaster master clock. The CYC-CLK heterodimer binds to the promoter region of per, causing transcriptional activation. PER and TIM can only accumulate in the cytoplasm in darkness because TIM is highly light sensitive. TIM and PER are transported to the nucleus, where they bind to CYC-CLK, preventing further transcriptional activation. This way, tim and per regulate their own expression. The levels of TIM-PER and CYC-CLK cycle reciprocally during the days, corresponding the activity of the fly.

The behaviour of *Drosophila suzukii*

D. suzukii is an invasive pest species. It causes significant losses for agriculture by laying its eggs inside ripening fruits. Originally from Asia, it has rapidly spread to Europe and America in the last 12 years, demonstrating high behavioural plasticity (Ørsted and Ørsted, 2019). The drastic seasonal phenotypic changes observed in fly populations are crucial in the adaptation to these new environments. *D. suzukii* switches between summer and winter morphs during the year. The winter morph is characterised by darker pigmentation and longer wings, which increases cold tolerance, allowing the fly to overwinter in colder temperate regions. Gene expression studies show that *D. suzukii* flies induce reproductive diapause in winter, while increasing carbohydrate metabolism and cuticular protein production (Shearer *et al.*, 2016). Their olfactory preferences also show seasonal changes (Clymans *et al.*, 2019). Interestingly, the circadian clock genes *tim* and *per* appear to be upregulated in winter morphs (Shearer *et al.*, 2016). However, whether these seasonal physiological adjustments are controlled by the circadian clock remains unknown.

D. suzukii carries a *D. melanogaster*-like master clock (Hansen *et al.*, 2019). Due to the lack of genetic tools available, the study of the *D. suzukii* circadian clock has so far been restricted to behavioural experiments on wild type flies (Hamby *et al.*, 2013; Hansen *et al.*, 2019). *D. suzukii* is reported to be primarily diurnal but not very active under laboratory conditions, although the activity patterns of different fly strains are variable. Overall, the circadian control of locomotion appears to be less robust in *D. suzukii* than in *D. melanogaster* (Hansen *et al.*, 2019).

The CRISPR/Cas9 system

The generation of *D. suzukii* clock mutants makes it possible to explore whether the circadian clock modulates seasonal behaviours. *tim*, a crucial and well-studied clock gene is an obvious candidate for mutation, while the CRISPR-Cas9 system is an ideal tool for transgenesis. The HDR-mediated CRISPR-Cas9 approach used in this project has been successfully employed on several insect species including *D. suzukii* flies (Li and Handler, 2017; Ahmed, Hildebrand and Wimmer, 2019). By combining the CRISPR

system with a donor plasmid, this technique allows specific insertion of exogenous markers into the genome while disrupting the targeted gene.

Aims

The primary aim of this project is to generate donor plasmids to disrupt the *tim* gene of *D. suzukii* via CRISPR-Cas9. Specifically, we focus on the sequencing of the *tim* locus of a wild type *D. suzukii* strain and cloning the homology arms into the pHD-RFP-attP and pHD-eGFP-attP donor plasmids. The project also aims to create a dataset to describe the circadian behaviour of *D. suzukii* through locomotor assays on wild type *D. melanogaster* and *D. suzukii* flies in solitary and pairwise settings.

Methods

Generation of donor plasmids

Cloning approach

The pHD-RFP-attP donor vector described by Gratz *et al.* (2014) was adapted to disrupt the *D. suzukii tim* gene via HDR-mediated CRISPR-Cas9 transgenesis. Two ~1kb long arms homologous to the genomic *tim* sequences will be cloned into the plasmid – this homology arm size have been shown to mediate efficient HDR (Beumer et al., 2013). The donor plasmid will contain one of two fluorescent marker genes, RFP or eGFP. The marker, positioned between the homology arms, will be inserted into the fly genome through HDR. Controlled by the hsp70 promoter and 3xP3 enhancer sequences, it will achieve strong expression in the eyes, making the rapid identification of mutants possible.

To generate *tim* knockout mutants by CRISPR, we designed two distinct strategies (Figure 2). The first approach employs two single guide RNAs (sgRNAs 1. and 2.), as recommended by Gratz *et al.* (2015). The pHD-RFP-attP (Addgene #51019) and pHD-eGFP-attP donor vectors will contain homology arms P1 and P2. Through double HDR, a 1.107kb region of *tim* will be replaced by the marker gene. The second approach uses one sgRNA (sgRNA 3.) – a single cut has also been shown to mediate efficient HDR (Auer *et al.*, 2020). The donor vector in this case will contain P2 and P3 homology arms, leading to the insertion of the marker gene into the *tim* sequence.

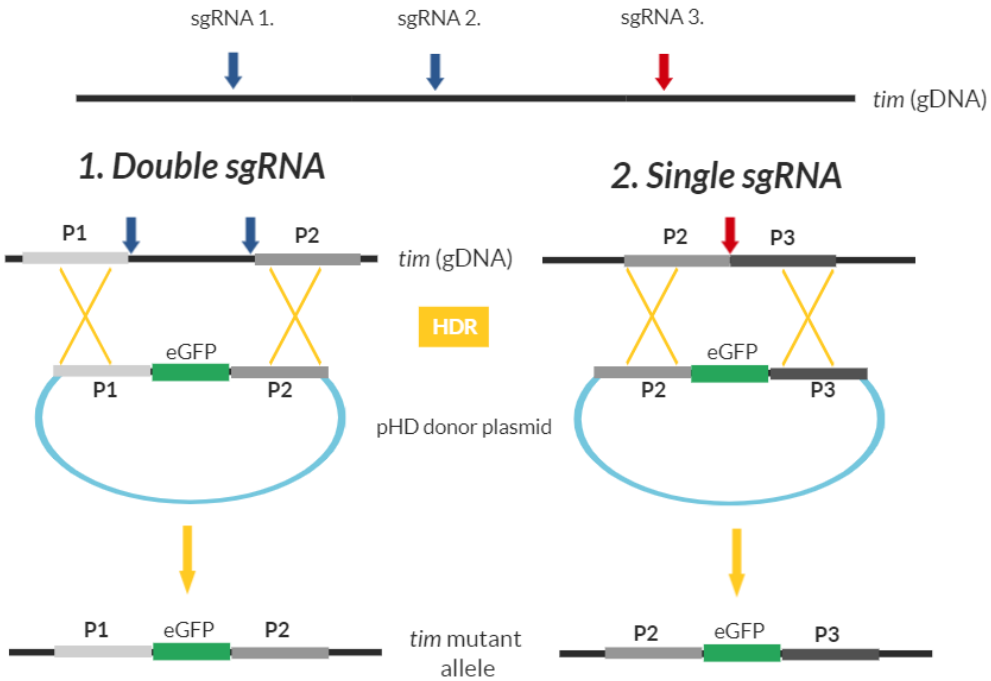

Figure 2: The two approaches (single and double sgRNA) used for transgenesis showing the 3xP3-hsp70-eGFP marker as an example. Inversion of the P1-2 homology arms is not shown for simplicity.

As recommended by Gratz *et al.* (2014), SapI will be used to clone the left (3') homology arm and AarI to clone right (5') homology arm. P2 is supposed to serve as the 3' homology arm in the first approach (Figure 2), but the presence of a SapI restriction site in P2 makes its cloning by SapI impossible. To tackle this problem, the reverse complementary sequence will be used for the cloning of P1 and P2. This way, the left and right arms will essentially be inverted, and AarI can be used to clone P2. To avoid digestion by SapI, P2 will be cloned into the vector after cloning of the other arm in both approaches.

1. Sequencing of the *tim* genomic region

Genomic DNA extraction

Genomic DNA was extracted from ~20 female *D. suzukii* flies (Quick-DNA Miniprep Kit, Zymo Research). The strain we used was established in 2017 from a collection of female flies in Oellingen, Germany.

Amplification of tim sequences

In order to know the precise sequence of a portion of *tim* in our *D. suzukii* strain, primers were designed to amplify three overlapping ~1.5kb long DNA fragments (1-3) based on the reference *D. suzukii* genomic sequence reported by Chiu *et al.* (2013). Primers were synthesised (Merck KGaA, Germany) and used in a PCR reaction (30sec 98°C - 40 cycles: 15sec 98°C, 15sec 58°C, 1min 72°C – 10min 72°C) with Phusion DNA Polymerase (Thermo Scientific, USA) (Table 1). Results were analysed on 1% agarose gel. Bands of the expected size were cut and extracted from the gel (NucleoSpin Mini kit, MACHEREY-NAGEL, Germany). Nucleic acid concentrations were quantified in NanoDrop 1000 spectrophotometer. sgRNA probes were also selected at this stage as predicted by ChopChop (Labun *et al.*, 2019), to ensure that the sequenced portions contained suitable target sites (Table 1).

***tim* fragment**	**sgRNA probe**	**primers**
1 (1266bp)	ACAGCAAATCCAGCGGG ACGAGG *ACGATCGACAGCAAATC CAGCGG	*tim*.suz1F: CCTTCTCCTCCTTAGGTTGTCT *tim*.suz1R: CTGAAATTCAAGGGTGGTAATC
2 (1405bp)	ACTGAGCTACTTGACCT ACGAGG TTACAAGTCCCACGTGT CGGCGG	*tim*.suz2F: AAGAGGAATACCTGCGACTAGG *tim*.suz2R: CTCAAATAGGGCTTCAGATCAC
3 (1983bp)	ACTGAGCTACTTGACCT ACGAGG	*tim*.suz3F: GGTACCCATAGATACATCCCAC *tim*.suz3R: GTGTCCATTTCCTCCTGAG

*Table 1: sgRNA target sequences and primers for the amplification of tim fragments. PAM sites (underlined) and Cas9 cuttings sites (space) are shown. * indicates another P1 target sequence chosen after a we detected a mutation that created a new PAM site.*

Cloning of tim sequences into the pJET vector

The fragments were ligated into the pJET plasmid following the CloneJET PCR cloning kit blunt-end cloning protocol (Thermo Scientific, USA). The resulting vectors were transformed into Stellar competent cells, plated on Amp^+ selective media and incubated overnight following the manufacturer's instructions (Clontech Laboratories, USA). pJET contains an Amp-resistance gene and a lethal restriction enzyme gene that is disrupted by ligation of an insert. Therefore, only bacteria containing the plasmid with an insert formed colonies. Liquid cultures were set in LB-Amp^+ medium from the bacterial colonies and incubated overnight in a shaking incubator. The plasmids were purified and eluted in 50µl H_2O following the Invisorb Spin Plasmid Mini Two protocol (Invisorb, Germany).

Sequencing of pJET inserts and identification of correct clones

The plasmids were digested with BglII (Thermo Scientific, USA) and analysed on 1% agarose gel to verify the size of the inserts (Figure 3). Samples with the insert of the correct size were used for further experiments. Samples were sequenced (Eurofins Genomics, Europe) using the primers JET_F:

CGACTCACTATAGGGAGAGCGGC and pJET_R: AAGAACATCGATTTTCCATGGCAG. The resulting sequences were aligned to the reported *D. suzukii* genomic DNA using Unipro Ugene (Okonechnikov *et al.*, 2012). The target sequence for fragment 1 was modified after the detection of a substitution. In fact, a new target was recommended by ChopChop that used the PAM site created by the substitution detected (Table 1).

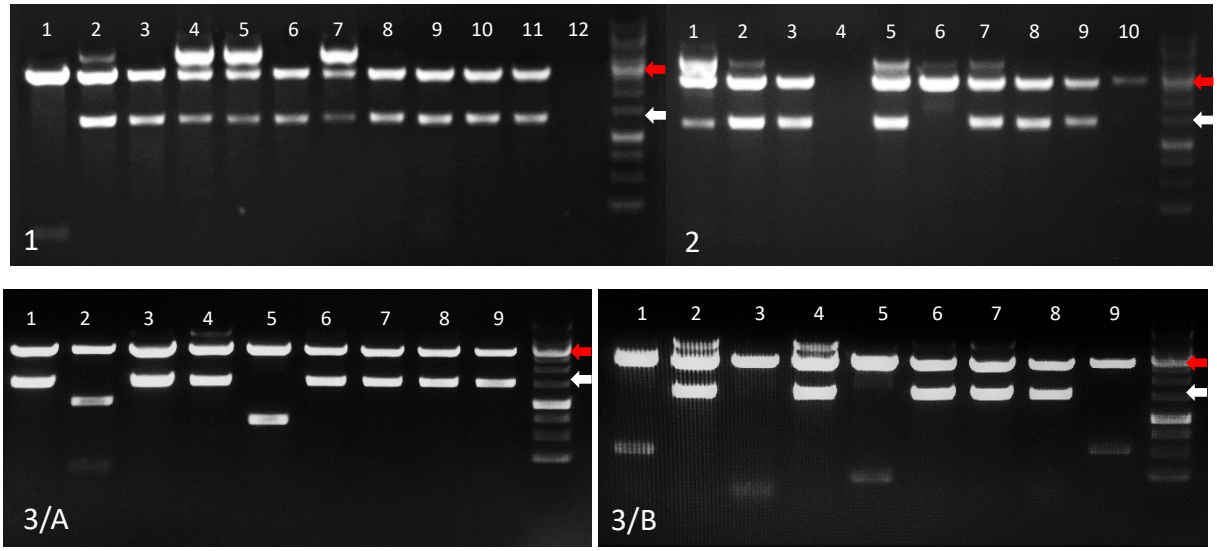

Figure 3: Result of the digestion of pJET plasmids with tim 1-3 inserts using BglII. GeneRuler 1 kb DNA Ladder (Thermo Scientific, USA) was used to assess the length of the fragments. Expected size for the tim sequence (~1.5kb) is indicated by red arrows. Expected size forthe pJET plasmid (~3kb) is indicated by white arrows. Lanes showing bands of the expected size: P1: 3,6,8-11; P2: 3,8,9; P3/A:1,3,6-9; P3/B:6-8.

2. Cloning of homology arm P1 into the pHD-RFP/eGFP-attP plasmid

Amplification of homology arms

Primers to introduce AarI and SapI restriction sites to the ends of the *tim* genomic sequences amplified previously were designed based on Gratz *et al.* (2015) (Table 2). The PCR reaction, gel electrophoresis and gel extraction were performed as described previously.

Approach 1	Left arm	SapI P1F: ccgtGCTCTTCg*TAT*TCCATGGACTCGCCATTGGG Sap P1*F: ccgtGCTCTTCg*TAT*GATTTGCTGTCGATCGTCATAATC SapI P1 R: ctagGCTCTTCt*GAC*GGACCAAACGCTTCGCAC
	Right arm	AarI P2 F: caatCACCTGCattc*TCGC*AGCTATCGGATTGCGGCTATG AarI P2 R: gactCACCTGCtcga*CTAC*AGGTCAAGTAGCTCAGTACATCG
Approach 2	Left arm	AarI P2 F.2: caatCACCTGCattc*TCGC*AGCTATCGGATTGCGGCTATG AarI P2 R.2: gactCACCTGCtcga*CTAC*AGGTCAAGTAGCTCAGTACATCG
	Right arm	SapI P3 F: ccgtGCTCTTCg*TAT*ACGAGGGTGTGTCCCTCTG SapI P3 R: ctagGCTCTTCt*GAC*TGGACATCCACTGATGGAGG

Table 2: Primers to introduce restriction sites required for the cloning of left and right homology arms in the two cloning approaches. Primers include the AarI/SapI restriction sites (underlined), spacer regions that ensure efficient cleavage and protect from degradation (lower case), cohesive ends generated by AarI/SapI digestion (italics) and ~20 bps complementary

to the homology arms. Forward primers were designed to anneal at the end of the Cas9 cutting site. Reverse primers were selected to make the final product ~1kb.

Cloning of P1 into the pHD-RFP/eGFP-attP plasmid

Both the pHD-RFP/eGFP-attP plasmid and the P1 PCR products were digested with SapI overnight at 37°C (Thermo Scientific, USA). Gel electrophoresis and gel extraction were performed, and concentrations were quantified as described previously. Ligation was performed following the manufacturer's protocol for T4 DNA ligase (Thermo Scientific, USA). Cell transformations and minipreparations were performed a described previously.

Identification of correct pHD-P1-RFP/eGFP-attP plasmids

To check if the plasmids contained the inserts of the expected size, samples were digested with BamHI (Thermo Scientific, USA) and analysed on 1% agarose gel (Figure 3). Sequencing of samples showing the expected pattern confirmed that they contained the P1 insert in the correct orientation, resulting in the final donor plasmid shown in Figure 4.

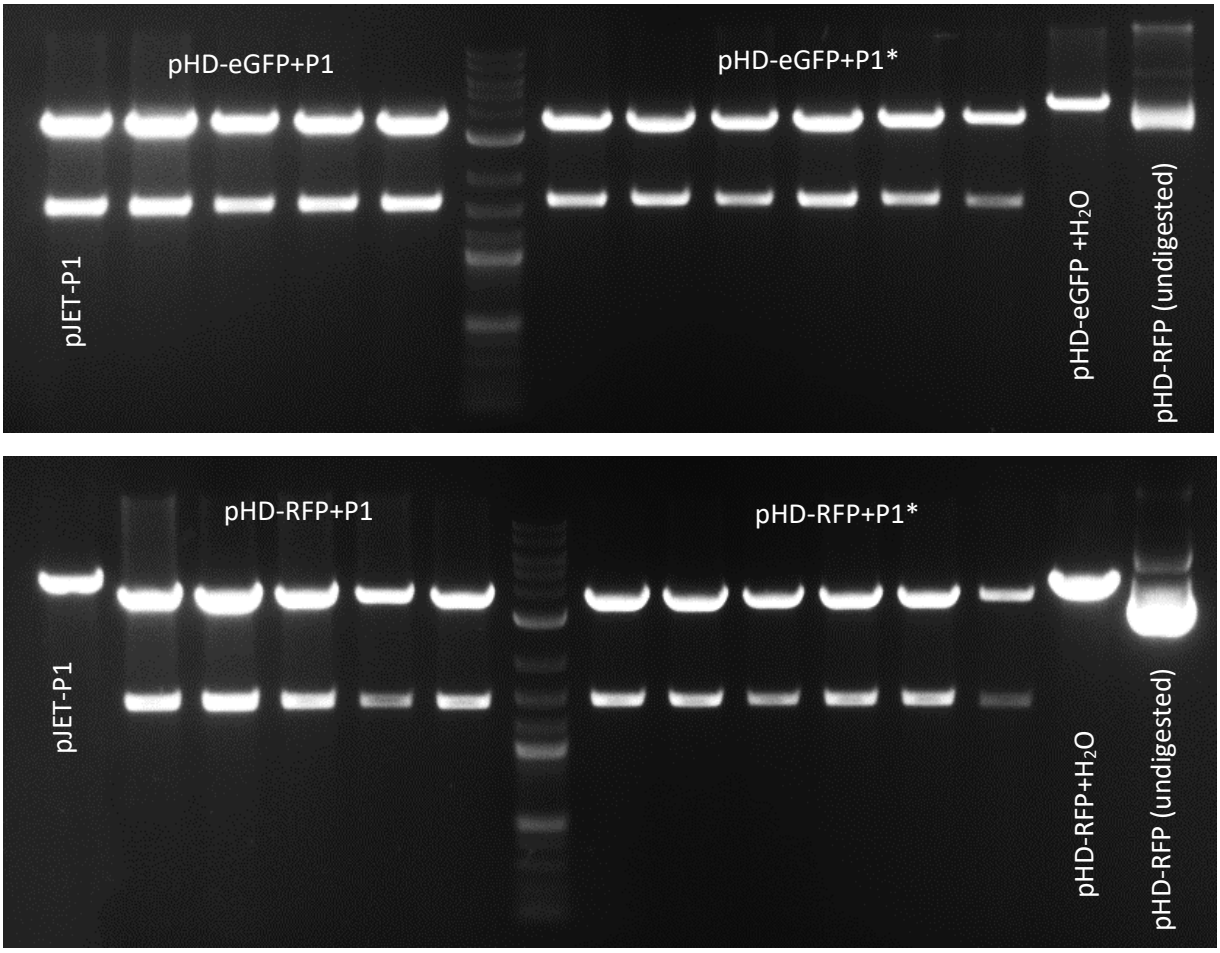

Figure 3: Results of the digestion of pHD-P1/P1-RFP/eGFP-attP plasmids with BamHI (performed by Damiano Zanini). BamHI had one restriction site in the plasmid and one in the P1 insert. Therefore, it produced two bands (3.5kb; 1.5kb) if P1 was successfully inserted. Controls included pJET-P1, the pHD plasmid digested without the insert, and the undigested plasmid.*

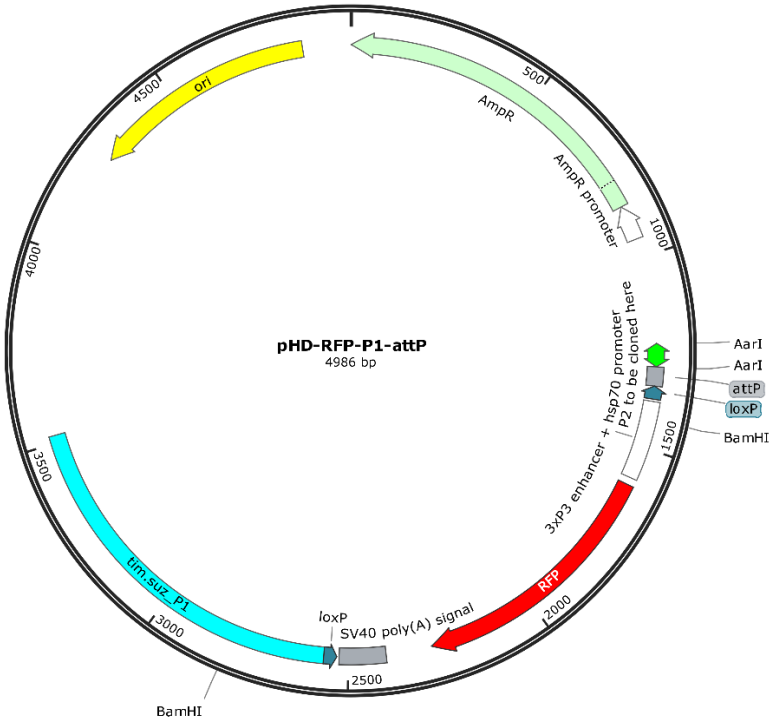

Figure 4: Map of the resulting pHD-RFP-P1-attP donor plasmid showing functional features, BamHI restriction sites used for the identification of correct plasmids, and AarI restrictions sites to be used later for the cloning of P2.

Locomotion experiments

Fly strains and maintenance

Wild type *D. melanogaster* (Lindelbach, Germany) and *D. suzukii* (Oellingen, Germany) flies were used for the locomotor assays. Flies were kept in groups at room temperature under natural light-dark cycles and were fed on yeast cornmeal media.

Locomotor assays

For individual experiments, 1-7 days old male flies were anesthetized by CO_2 and transferred into 5mm glass tubes containing ~1.5cm agar-sucrose media. Male flies are preferred over females, as the behaviour of females may be affected by oviposition and the emerging larvae. For the pairwise experiments, same- and mixed-sex pairs were selected and transferred into 7mm tubes. Tubes were sealed with cotton plugs and inserted into 32-tube *Drosophila* activity monitors (DAM2, LAM25, Trikinetics, USA) (Figure 5). The monitors measure the number of crosses the flies make each minute based on the disruption of an infrared light beam. All experiments were carried out in light-controlled incubators at 22°C. Following 6-7 days of 12h:12h rectangular (on-off) light-dark entrainment (LD), flies were recorded for 10-12 days in complete darkness (DD).

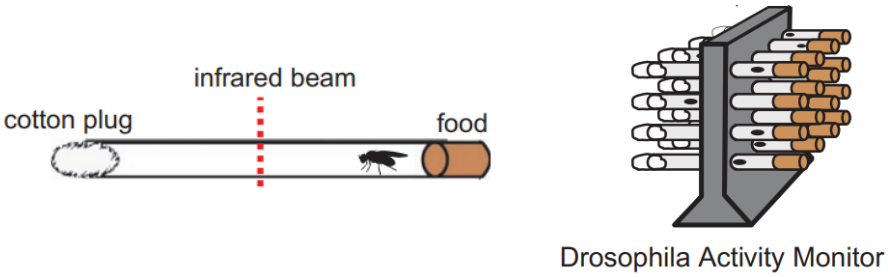

Figure 5: Experimental setup of Drosophila locomotor assays. The tubes detect disruption of infrared light beams in the centre to assess locomotory activity. Figure adapted from Kirszenblat and van Swinderen (2019) *FIG. 22.1.*

Statistical analysis

The circadian period analysis was performed manually using the ActogramJ software (Schmid, Helfrich-Förster and Yoshii, 2011). Rhythmicity under DD was determined based on the presence of subjective evening peaks, using Lomb-Scargle periodograms. ActogramJ and ShinyR-DAM (Cichewicz and Hirsh, 2018) were used to create the graphs. Tubes containing dead flies were excluded from the analysis.

Results of the locomotor experiments

Single fly locomotor activity

Our single fly locomotor assay followed the standard protocol of recording under DD conditions after a few days of 12h-12h rectangular LD entrainment (Rosato and Kyriacou, 2006). As expected, *D. melanogaster* flies were highly active and displayed characteristic crepuscular activity patterns with pronounced morning (M) and evening (E) peaks (Figures 6-7). *D. suzukii* were significantly less active. Nevertheless, they showed a bimodal activity pattern under LD. Consistent with the findings of Hansen *et al.* (2019), *D. suzukii* flies showed increased activity only after the light switch, while *D. melanogaster* exhibited strong anticipation leading up to the peaks. While all *D. melanogaster* flies maintained their rhythmicity under DD, 17% of *D. suzukii* flies became completely arrhythmic (Table 3). The statistical analysis detected periodicity in the activity of most *D. suzukii* flies, but many of them showed complex activity patterns. In addition, rhythmic *D. suzukii* showed much higher variation in period length and a longer average period (Table 3). Individual actograms revealed visible though variable E peaks and generally diurnal activity in rhythmic *D. suzukii* under DD conditions (Figure 7).

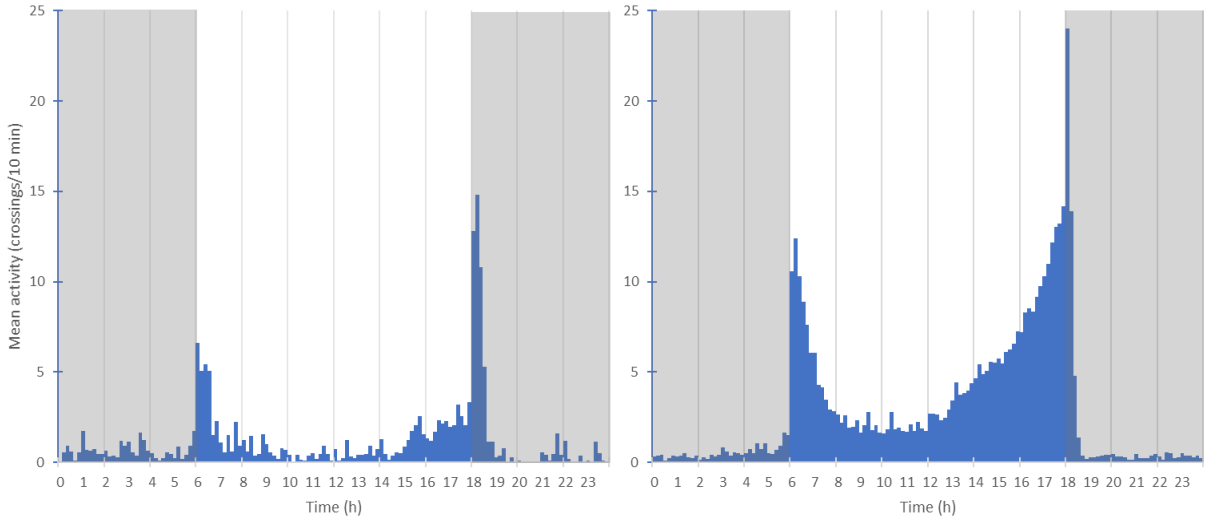

Figure 6: Mean activity profile of D. suzukii (left) and D. melanogaster (right) flies based on 6 LD days. The grey and white backgrounds correspond to darkness and light.

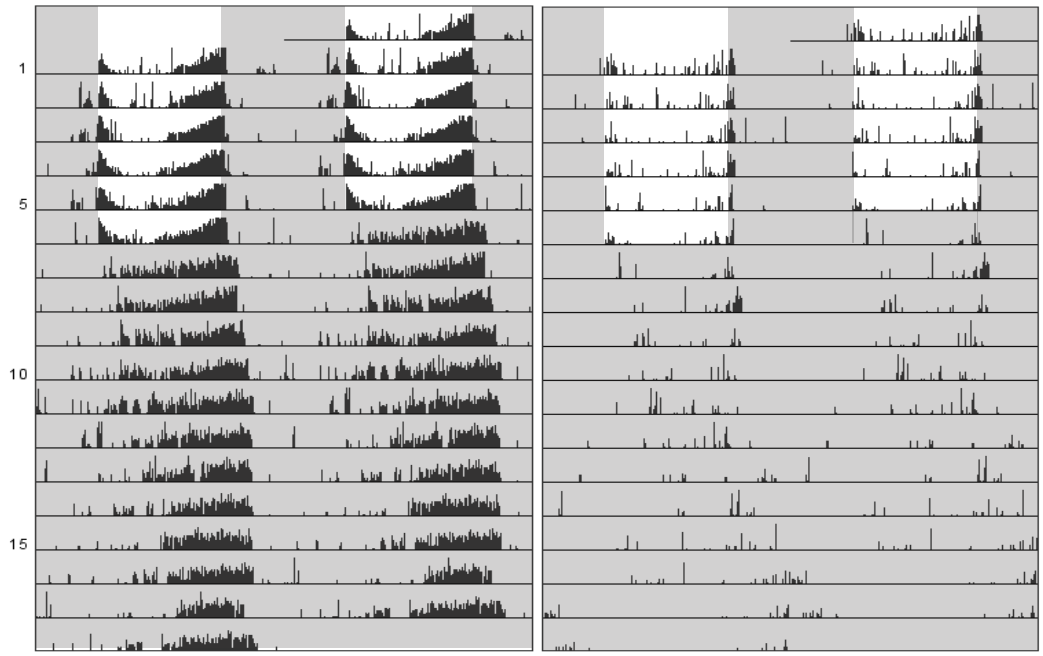

Figure 7: Double plotted actograms representing the activity of a single rhythmic D. melanogaster (left) and D. suzukii (right) fly under LD and DD conditions (x axis: time; y axis: activity (crossings/min) on each experimental day). The rightward shift during the DD days in D. melanogaster indicates that the free-running period length of this individual is slightly over 24 hours.

	R	C	A	τ [R] (h)	S.dev [R]
D. melanogaster	100%	0%	0%	23.7	0.28
D. suzukii	69%	14%	17%	25.0	1.19

Table 3: Locomotor activity of n=30 D. melanogaster and n=29 D. suzukii flies tested in single fly locomotor assay. R: number of flies rhythmic under DD; C: number of flies showing complex activity period under DD; A: arrhythmic flies under DD; τ [R] (h): circadian period of R flies (h); S.dev [R]: standard deviation of τ [R].

Locomotor activity in pairs

The inactivity of *D. suzukii* flies makes it difficult to explore the potential circadian control underlying their behaviour. As they have been shown to exhibit higher activity in social settings (Hansen *et al.*, 2019), we ran pairwise locomotor assays. Our results show similar patterns to individual assays, but a lack of arrhythmic flies was found (Table 4, Figure 8). Moreover, in these experiments M and E activity peaks were more difficult to detect under DD, and a more general diurnal activity pattern emerged in both species (Figure 9). Consistent with previous findings (Fujii *et al.*, 2007), the mean LD activity of *D. melanogaster* mixed sex pairs revealed high activity during the night as well as the day (Figure 9). Our results show a lack of such changed locomotory behaviour in *D. suzukii* male-female pairs. Male-male pairs show somewhat more pronounced peaks and daytime 'siesta' compared to other sex combinations in both species.

Pairings	Spp.	R	C	τ [R] (h)	S.dev [R]
M-M	*D. mel*	100%	0%	23.7	0.32
	D. suz	75%	25%	24.9	0.80
F-F	*D. mel*	90%	10%	24.2	0.15
	D. suz	83%	17%	24.2	0.41
M-F	*D. mel*	82%	18%	24.2	0.56
	D. suz	82%	18%	24.7	0.86

Table 4: Locomotor activity of D. melanogaster and D. suzukii flies in pairwise locomotor assays based on n=9-12 flies per pairings that survived the experiments. f-f: female-female pairs; m-f: mixed pairs; m-m=male pairs; R: number of pairs rhythmic under DD; C: number of pairs showing complex activity period under DD; τ [R] (h): circadian period of R pairs (h); S.dev [R]: standard deviation of τ [R]. No arrhythmic fly pairs were found.

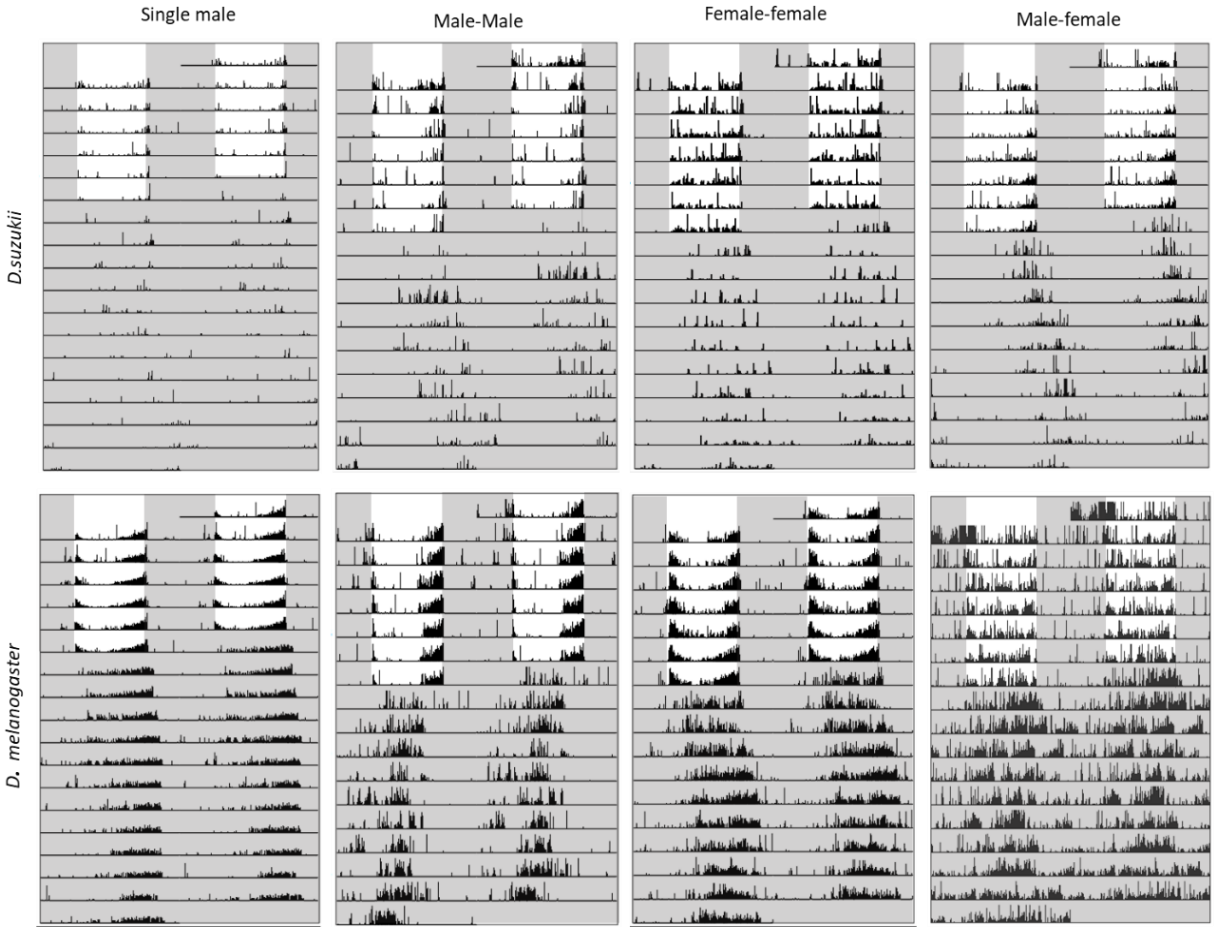

Figure 8: Individual actograms showing the results of pairwise experiments in the two species and sex combinations. A representative actogram of the single fly experiment is shown for comparison. All graphs are to scale.

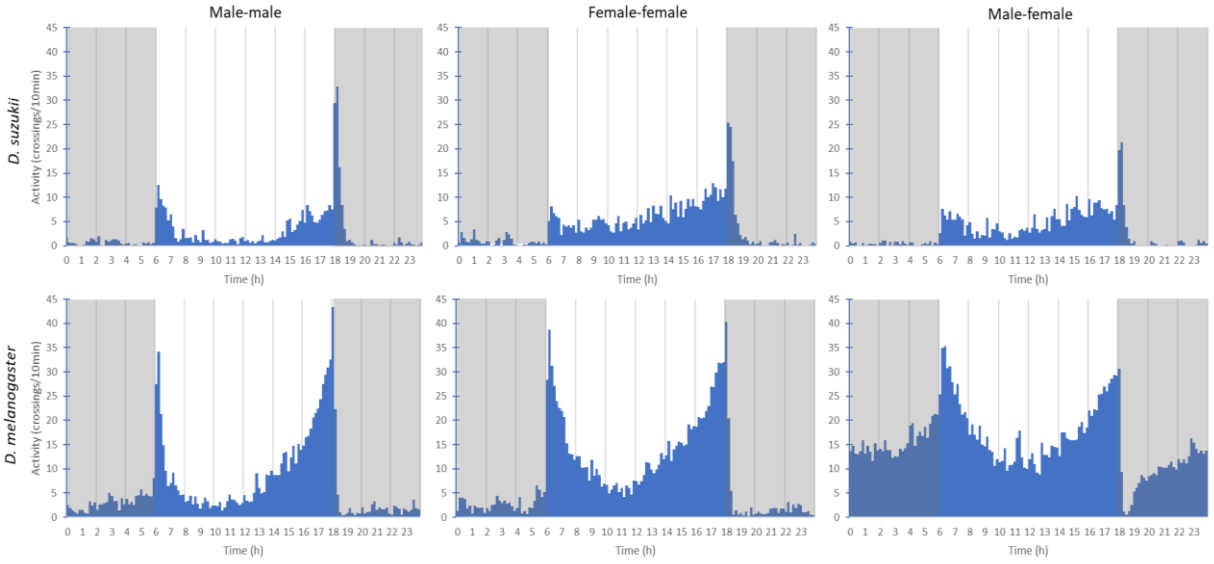

Figure 9: Mean activity of male-male, female-female and male-female D. suzukii and D. melanogaster pairs on LD days. Each graph was produced based on 6 days and 9 randomly chosen pairs.

Discussion and future steps

Transgenesis

As a next step, homology arms P2 and P3 will be cloned into the pHD-P1-RsRed/eGFP-attP plasmid, completing the generation of donor plasmids. Wild type *D. suzukii* embryos will be injected with the donor plasmid, sgRNA and Cas9 source. Mutant will be selected based on the visual marker, and the presence of the correct mutation will be verified by sequencing. An analogous donor plasmid for the mutation of *per* in *D. suzukii* has recently been created. Thanks to the use of two distinct visual markers, easy identification of *tim-per* double mutants may be possible in future. The circadian behaviour of the resulting fly lines will be analysed in locomotor experiments.

Expected behaviour of *tim* knockout *D. suzukii*

D. melanogaster tim mutants have been extensively studied, providing a basis for predictions on the behaviour of *tim* knockout *D. suzukii* flies. Both *tim* and *per* null mutant *D. melanogaster* are arrhythmic in terms of locomotor activity and eclosion under DD (Konopka and Benzer, 1971; Hardin, Hall and Rosbash, 1990; Sehgal *et al.*, 1994). In the *tim* null mutant the lack of functional TIM disrupts the characteristic daily cycling of both TIM and PER, because TIM is essential for the stabilisation of PER (Peschel and Helfrich-Förster, 2011). As the circadian clock genes are highly conserved, *tim* knockout *D. suzukii* flies are also expected to have seriously impaired circadian functions. The disruption of the master clock in these mutant lines will allow us to explore the possible circadian control behind seasonal changes in *D. suzuki* The clock genes *tim* and *per* have been found to be upregulated in winter *D. suzukii* morphs (Shearer *et al.*, 2016). Studying the *tim* and *per* knockout lines could ascertain whether clock genes are indeed involved in seasonal physiological adjustments – if the seasonal phenotypes of mutants are disrupted, this could serve as evidence for circadian involvement.

Locomotor experiments

The set of locomotory data created in this project describes the activity pattern of the Oellingen *D. suzukii* line. As the activity patterns of *D. suzukii* strains collected at different locations varies considerably (Hansen *et al.*, 2019), it was important to determine the circadian activity of the line that will later be used for transgenesis.

Our findings suggest that *D. suzukii* flies might be able to interpret photoperiodic information less precisely than *D. melanogaster*. This is supported by the large number of arrhythmic *D. suzukii* flies under DD conditions (Table 3). Anticipation leading up to activity peaks, which is an important sign of the endogenous nature of circadian behaviour as opposed to a simple reaction to light (Sheeba, Fogle and Holmes, 2010), was less pronounced in *D. suzukii*. The period length of *D. suzukii* flies was also found to be less consistent. However, the general inactivity of *D. suzukii* flies makes the determination of periodicity less robust, making these findings less conclusive.

The *D. suzukii* line observed appears to be primarily crepuscular under laboratory conditions. Although less consistently than *D. melanogaster*, most *D. suzukii* flies also displayed E activity peaks, and were almost completely inactive during the subjective night. These results are not fully consistent with Hansen *et al.* (2019), which suggests that some *D. suzukii* lines are more diurnal than bimodal. Climatic factors could potentially account for these differences, as Hansen *et al.* (2019) used flies collected in Italy, Trento. However, it observed significant variation even between the locomotor behaviour of lines derived from the same collection. In addition, experimental temperatures might also affect the presence of activity peaks in *D. suzukii* (Hansen *et al.*, 2019; Shaw, Fountain and Wijnen, 2019).

There is some evidence that the differences in the activity patterns of *D. melanogaster* and *D. suzukii* flies might result from the neural organisation of their master clocks (Hansen *et al.*, 2019). However,

these slight differences cannot fully explain this phenomena and further investigations are needed. The distinct activity level of the two species could be explained from a behavioural perspective. The relatively large number of tubes containing dead *D. suzukii* flies (12%) suggests that the experimental conditions were highly stressful. It has been suggested that the two species use opposite strategies to cope with stress – while *D. melanogaster* reacts with increased activity, *D. suzukii* become inactive to save energy until more favourable conditions arise. Switching of the light might have triggered a startle response in *D. suzukii* flies causing inactivity, potentially masking the true extent of circadian behaviours. This issue could be avoided by running experiments in seminatural conditions.

Pairwise settings could also help overcome the low activity of *D. suzukii* flies in locomotory assays. *D. suzukii* have been shown to be more synchronous and active in groups, suggesting that social cues are important Zeitgebers of the circadian clock (Hansen *et al.*, 2019). Social synchronisation has been observed in *D. melanogaster* flies, and it appears to be mediated by olfaction (Lone and Sharma, 2011). Although our experiments did not allow direct comparison between activity levels in single fly and pairwise assays, they revealed a lack of arrhythmic *D. suzukii* flies in pairwise experiments in contrast with individual assays. However, further evidence is needed to determine the extent of social synchronisation in *D. suzukii* in pairwise settings.

Pairwise experiments suggest that the sex composition of pairs is an important determinant of circadian behaviour. Fujii *et al.* (2007) observed distinct locomotory behaviour in heterosexual *D. melanogaster* pairs in close-proximity assays. Our results show that the same patterns are present in simple pairwise locomotory assays – Figure 9 reveals that male-female couples are active throughout the day and night under LD conditions, except for a brief rest phase at dusk. This is due to the circadian controlled courtship behaviour exhibited by male flies (Fujii *et al.*, 2007). Males appear to chase females even after copulation, resulting in constantly high activity levels (Fujii *et al.*, 2007). Our experiments have shown no similar change in activity in mixed sex *D. suzukii* flies.

Some degree of caution must be applied when interpreting the results of the pairwise experiments, as they do not allow the identification of individual flies. This makes it impossible to account for the differential contribution of the two flies to the activity level registered by the monitor. Moreover, other rhythmic behaviours might interfere with locomotory data in pairwise settings, including courtship, mating, aggression and feeding. To tackle this problem, pairwise experiments could be performed in different settings that allow the identification of individual activity levels (e.g. video recording).

D. suzukii is a promising organism to study the role of seasonal adaptation in the context of the circadian clock. This project resulted in the creation of donor plasmids to be used in HDR-mediated CRISPR-Cas9 transgenesis of *D. suzukii* flies. Additionally, our locomotor experiments described the activity patterns of *D. suzukii*. Our strain was highly inactive and appeared to have a less robust circadian clock compared to *D. melanogaste*r. *tim* knockout *D. suzukii* lines are expected to have impaired rhythmic behaviours, making it possible to investigate the circadian control of seasonal phenotypic changes in future.

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