# Circadian Fluctuation in Fly Immune Defense

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## Certificate of Examination

This is to certify that the dissertation titled "**Circadian fluctuation in fly immune defense**" submitted by **Ms. Kimaya Tekade** (MS16063) for the partial fulfillment of BS-MS dual degree program of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

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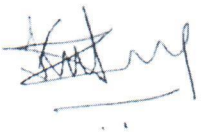
Dated: April 9, 2021

## Declaration

The work presented in this dissertation has been carried out by me under the guidance of Prof. N. G. Prasad at the Indian Institute of Science Education and Research, Mohali.
This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.



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Dated: April 9, 2021

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Prof. N. G. Prasad
(Supervisor)

# Acknowledgement

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# ABSTRACT

We investigated if the immune response in *Drosophila melanogaster* varies as a function of time-of-infection, and if yes the does this rhythm change in flies which have been selected for improved post-infection survivorship for over 70 generations. To investigate this, we used an outbred selection line from the lab- EPN. We checked this phenomenon in both the sexes. Our results show that there isn't any robust circadian pattern in determining mortality both in males and females. Although it was observed that female flies are more resistant to night-time infections than infections done during daytime.

# INTRODUCTION

The physical environment of earth is highly rhythmic. Many rhythms are caused by geophysical cycles, including diel, tidal, lunar, and annual rhythms. Over an evolutionary time scale, organisms across kingdoms have adapted to these fluctuations by ensuring to have an internal representation of time which keeps track of time even under constant conditions. The most obvious of all environmental fluctuations is the 24-hr cycle of dark and light which results due to the rotation of earth. These rhythms are predictable and have resulted in the evolution of biological clocks in organisms of almost all phyla. An internal clock enables partitioning of organism's activities in suitable temporal niches. For example, the unicellular alga Gonyaulax polyedra is a marine dinoflagellete which thrives on the surface of the ocean during daytime for carbon fixation by photosynthesis and sinks at the bottom of the ocean at night to gather minerals such as phosphate, sulphate, nitrogen, etc. The dinoflagellete cells increase their density at night to be able to enter the deeper waters and emit light at the same time. Before the sun rises, they generate gas bubbles and start their journey upwards towards the surface. Gonyaulax polyedra has adapted to a physiology that can obtain energy as well as nutrients both of which are spatially and temporally separated. This behaviour is not just a mere response to day/light stimuli but is endogenous because it continues under laboratory constant conditions [1]. A biological clock is called circadian as it keeps track of length of time which is of about 24 hrs. The term 'circadian' comes from Latin words `circa' which means 'about' and 'diem' that means 'day` [2]. The period of an organism's rhythmic activity monitored under constant conditions is called the free-running period which is a bit more or less than 24hrs. It is crucial that the organism be able to entrain its clock to the local 24-hour cycles. Such entrainment occurs by environmental cues like light called 'zeitgeber'. An organism's circadian clock is defined as an endogenous and temperature-compensated mechanism [3], which runs with free-running period when kept in constant conditions but is normally susceptible to entrainment to the local 24-hour cycle.

Circadian rhythms are driven by an internal clock which is essentially a set of transcriptional and translational feedback loops. *Drosophila melanogaster* has proven to be a very useful model organism in investigating the genes which form the core of circadian clock. Although all the clock genes are not homologous across organisms of several phyla, the organization

and the basic mechanisms which generate rhythmicity are very similar. Konopka and Benzer, in 1971, first discovered a clock gene named 'period' by doing a forward mutagenesis screen in Drosophila melanogaster. Three mutants were isolated in which the 24-hr rhythm of eclosion and locomotor activity was drastically changed. One was an arrhythmic mutant, another long-period mutant whose period was found to be 28hr and a short-period mutant with a 19hr period. These mutations in period gene were mapped on to the X chromosome in flies [4]. Many more genes of the clock network have discovered since then and we now have a deeper understanding of the molecular clock in Drosophila melanogaster. According to the current model, the transcription factors CLOCK (CLK) and CYCLE (CYC) form a heterodimer and bind to the E-BOX region of DNA during the day [5, 6]. This region acts as a promoter for several clock genes like *period (per)* and *timeless (tim)*. This results in the accumulation of *per* and *tim* mRNA in the cytoplasm. Since TIMELESS protein is sensitive to light, it is only during the darkness that PER and TIM accumulate, form a heterodimer and enter the nucleus [7,8]. The PER/TIM complex then promotes the phosphorylation of CLK/CYC at night, which reduces the affinity of the CLK/CYC dimer for DNA [9,10]. This inhibits the transcription of *per* and *tim* genes and forms a negative feedback loop. Eventually, PER and TIM are also degraded by phosphorylation during the day, releasing CLK/CYC from repression and thus starting a new cycle all over again [11]. For further illustration, the reader can refer to the figure below taken from the review article '**Setting the clock – by nature: Circadian rhythm in the fruitfly *Drosophila melanogaster'*** by Nicolai Peschel and Charlotte Helfrich-Förster. I have explained the clock mechanism in the simplest possible way; there are obviously several other genes involved which aid the feedback loops. The proteosomal degradation of TIM followed by the resetting of the clock is an important event which requires light input. The clock receives light as an input through the activation of a blue light photoreceptor named CRYPTOCHROME (CRY) [12]. Upon photon absorption CRY undergoes conformational changes and binds to TIM. This induces degradation of TIM, thus resetting the clock [13,14]. In *Drosophila,* the circadian clock gates eclosion, courtship and determines the period of rest and activity [22, 23, 24, 25]. Besides these behaviours, the clock also coordinates many rhythms in peripheral organs such as olfactory and gustatory sensitivity rhythms [26, 27].

The circadian clock in mammals resembles the mechanism in Drosophila in its basic organization of positive and negative feedback loop and a few genes like *period*, *cryptochrome* and *clock*. However, there are some differences. In mammals, the

suprachiasmatic nucleus (SCN) has shown to be a circadian pacemaker [15, 16]. SCN contains several cell-autonomous oscillators which receive photic input through the retinohypothalamic tract (RHT), and are coupled to each other to set the phase and period of biological rhythms throughout the organism [17]. In mammals, the rhythmic expression of clock genes is not restricted to SCN but is widely distributed among peripheral cells and tissues, including liver, endocrine tissues, the heart and the skeletal muscles [18, 19, 20]. The molecular oscillators in these tissues are similar to that found in SCN, but these tissues do not receive direct photic input. This suggests a hierarchical model in which SCN receives light and provides the zeitgeber required to maintain synchronized internal circadian time keeping mechanism [18] In mammals, the transcription factors CLOCK (Circadian locomotor output cycles kaput) and BMAL1 (brain and muscle ARNT-like protein 1) for heterodimer and bind to the DNA. This activates the transcription of period (*per1* and *per2)* and cryptochrome (*cry1* and *cry2)*. PER and CRY slowly accumulate to feed back to inhibit CLOCK-BMAL1-dependent transcription [21]. In mammals, all aspects of physiology are regulated by internal circadian clocks, including sleep–wake cycles, behaviour and locomotor activity, body temperature cycles, cardiovascular and digestive processes, endocrine systems and metabolic and immune functions.

An often over looked aspect while dealing with host-pathogen interactions is the circadian state of the host. The ability to combat bacterial infections is an essential trait and the circadian variation in its immune response was highlighted long back in 1969. In this study, mice were infected with *Diplococcus pneumonia* at different times of the day (4:00, 8:00, 12:00, 20:00) and it was observed that mice infected at 4:00 showed higher survival rates than those infected at other time points [28]. Later in 1972, another experimental study showed that it took longer time for the bacteria to reach an intense level ($10^8$ bacteria/ml) in mice when infected at 4:00 than the time needed for mice to reach the same level of bacteria infected at 16:00 [29]. A ton of improvement has been made since these initial experiments, in understanding the role of clock in host-pathogen interactions including bacteria, viruses and parasites. F. Hawking in 1970 proposed that parasites benefit from adapting their time of maximal transmissibility to the time of the day the insect vector is foraging and thus is most likely is taken up by it [30]. There has been experimental evidence supporting Hawking. High nightly counts of *Wuchereria bancrofti*, a filarial nematode, have been observed in the blood of the host, which is presumably an adaptation to the night biting *Anopheles* and *Culex* vector. In contrast, the Pacific-type *Wucheria Bancrofti* peaks in its circulation in the

afternoon, consistent with the phase of the biting behaviour of *Aedes*, its diurnal (active during the day) vector [30, 31]. It is not yet known whether parasites themselves evolve endogenous time-keeping strategies.

The responsiveness, migration, localization, and activity of immune cells exhibit robust circadian rhythmicity [32]. Several clock genes are directly involved in immune functions. For example,*Bmal1-/-* mice exhibit marked deficits in B cell numbers and immunoglobulin titers [33] Laranjeira-Silva showed that infection of mice by *Leishmania* parasite during late day led to karger lesions than upon infection in late night [33]. Interestingly, the recruitment of innate immune cells such as neutrophils and macrophases which are the host cells for Leishmania also peaks during late day. The rhythms in parasite load, immune cell recruitment and chemokine expression were abolished in mice lacking circadian clock [34]. 'Circadian rhythms in Immunity' by Stephen N.Waggoner and 'Clocking into immunity' are two outstanding comprehensive reviews to know in details about this topic

Getting back to *Drosophila melanogaster*, the model of our study system, there have been a few studies providing evidence for circadian variation in anti-bacterial immunity in flies. The circadian gene expression in flies was studied using high density oligonucleotide arrays [35,36]. This led to the identification of 134 cycling genes involved in several physiological processes. Genes like *Immune deficiency (imd), u-shaped (ush)* and *acetylated low density lipoprotein receptor (ldlr*) which are involved in host defense were among the several cycling genes [35]. Hiza *et.al* in 2007 provided the first functional and bi-directional link between circadian rhythm and innate immunity in *Drosophila melanogaster.* It was observed that on infecting flies with gram positive bacteria *Streptococcus pneumonia* and *Listeria monocytogenes* flies move constantly and lose circadian regulation of locomotion. Sick flies also do not sleep well, they have fewer sleep bouts. Similar results were observed with $tim^{01}$ mutant flies. Mutant flies were also immunocompromised. $Tim^{01}$ and $per^{01}$ flies when infected with *Streptococcus pneumonia* and *Listeria monocytogenes* died significantly faster than wild-type flies [37]. This evidence suggested that circadian genes regulate innate immunity in *Drosophila.* Another study demonstrated that the time-of-day of infection affects the post-infection survival of flies. Flies were first entrained to a 12hr light/12 hr dark cycles (LD 12:12; where zeitgeber time 0 [ZT0] is defined as time of lights ON) for 2 days and then infected with gram negative *Pseudomonas aruginosa* and gram positive *Staphylococcus aureus* every 4 hour round the clock. It was observed that flies infected at ZT21survived

approximately 4-fold better compared to flies infected at ZT5. This held true for infections over a broad range of initial bacterial doses. Daily changes in survival rates of flies were also observed in DD proving that the survival rhythm is endogenously driven [38]. Also, no robust daily rhythms in survival were observed for different arrhythmic clock mutants $per^{01}$, $tim^{01}$, $Clk^{Jrk}$ and $cyc^{01}$. $Per^{01}$ flies showed higher mortality rate than control flies consistent with the results from previous Hiza *et.al* study, however this group observed that $tim^{01}$, $Clk^{Jrk}$, and $cyc^{01}$ mutants showed enhanced survivorship compared to control flies [38]. The reason behind this discrepancy is yet unclear but it might be due to the use of different bacteria for infection and its different mode of pathogenicity. Taken together, these studies indicate that flies survive night time infections better than daytime. Stone *et.al.* in 2012 tested the hypothesis that circadian mutants are sensitive to infection due to changes in mechanisms of resistance and investigated resistance mechanisms like antimicrobial peptide synthesis, reactive oxygen species (ROS) synthesis and phagocytosis for circadian regulation. To shed light on the previously described discrepancy, this group infected *tim* mutants with three other pathogenic bacteria- *Serratia marcescens*, *Burkholderia cepacia*, and *Salmonella typhimurium*. *Tim* mutants died more quickly than wild-type flies when infected with *S. marcescens* and died with wild-type kinetics when infected with *S. typhimurium* and *B. cepacia* [39]. These data suggests that circadian immune phenotypes are pathogen-specific. High bacterial load data from *tim* mutants infected with *S.pneumuniae* and *S.marcescens* suggests that *tim* mutants are less able to control microbial growth as compared to WT [39]. Thus, Timeless seems to regulate resistance mechanisms in pathogen specific manner. TIM protein levels oscillate over the day [40], and consistent with its expression levels it was observed that WT flies infected at ZT07 (lowest TIM expression) died faster than those infected at ZT19 (peak TIM expression) [39]. Insects react to injuries and some bacterial infections by generating toxic ROS and depositing melanin, visible through the cuticle as dark black spots [41]. It was found that there is no significant difference in the melanised spot formation between WT and *tim* mutant flies or between WT flies infected at different times of the day [39]. This tells that melanisation is not regulated by *tim*. It was also observed that AMP gene expression induced by septic injury is not circadian regulated. However, it was demonstrated that *tim* regulates bacteria specific phagocytosis by immune cells. pHrodo (pH sensitive rhodamine dye) labelled dead *S.aureus* bacteria were injected in flies at different times of the day, and it was observed that phagocytic activity was significantly higher at Z19 (night) than ZT07 in WT flies. This difference in phagocytic activity was not observed in infected *tim* mutants [39]. In contrast to *S.aureus*, WT and *tim* mutants both did not exhibit

circadian difference in phagocytosis of *Escherichia coli*. This suggests that TIM regulates a bacteria specific step of phagocytosis like substrate recognition or binding [39].

In the present study, we carried out experiments to investigate-

*I.* If there is a circadian variation in survivorship, on infecting outbred flies with gram-positive bacteria *Enterococcus faecalis*

*II.* If I is true, then how does this rhythm change in flies which have been selected for improved post-infection survivorship for over 70 generations. Refer to figure 1.2 for the proportion mortality observed after infecting selection line at 65$^{th}$ generation. One can observe that the mortality proportion of selected population (E) is approximately 50% less than control population (N)

*III.* Whether males and females respond differently to the infections

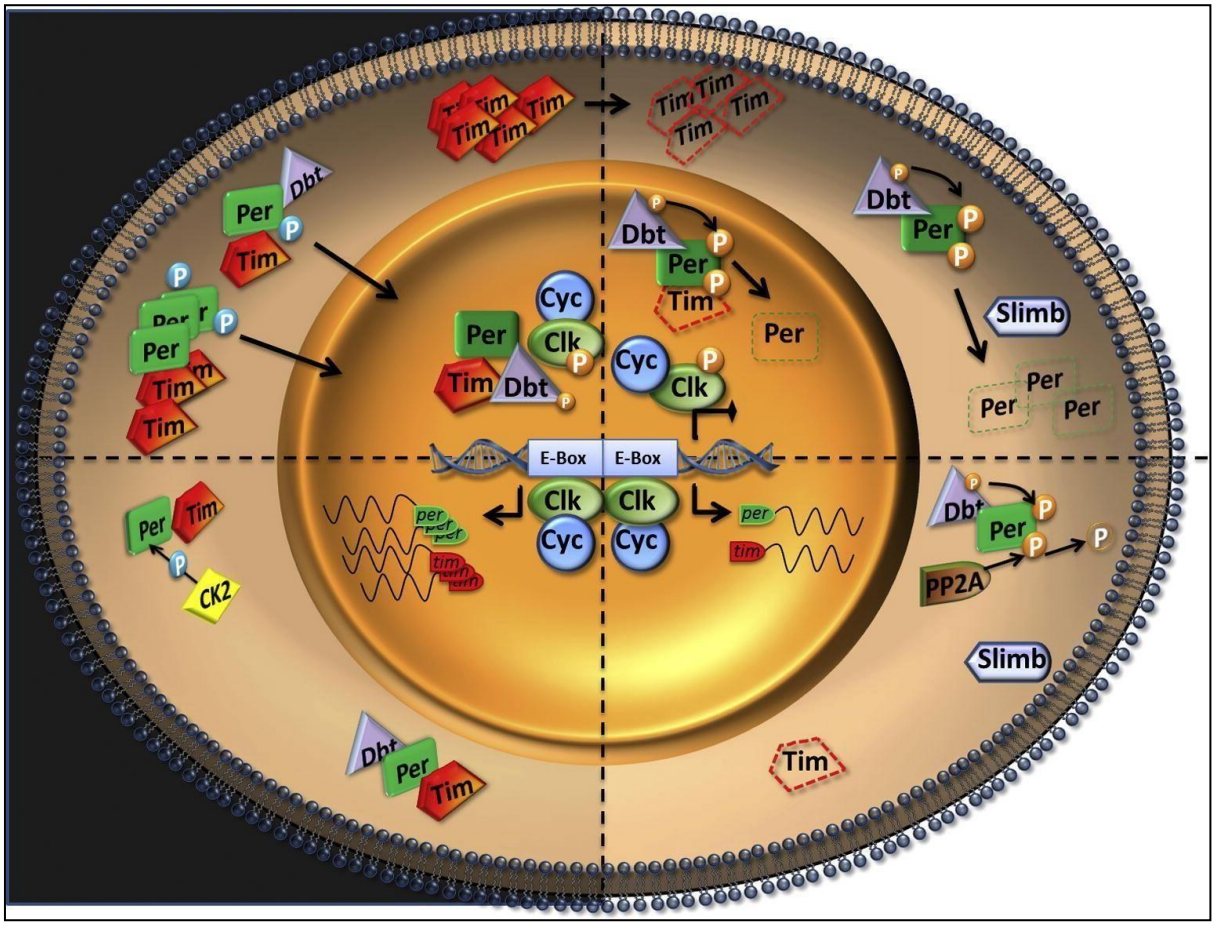


**Figure 1.1**: The molecular mechanism of clock in Drosophila melanogaster. The figure displays a clock cell at different times of the day. Each quarter of the circle represents 6hr of the circadian day. The right side indicates daytime and the left indicates night time. Dotted boundaries indicate proteasomal degradation. 'P' indicates phosphorous (Image source: N. Peschel, C. Helfrich-Förster / FEBS Letters 585 (2011) 1435–1442)

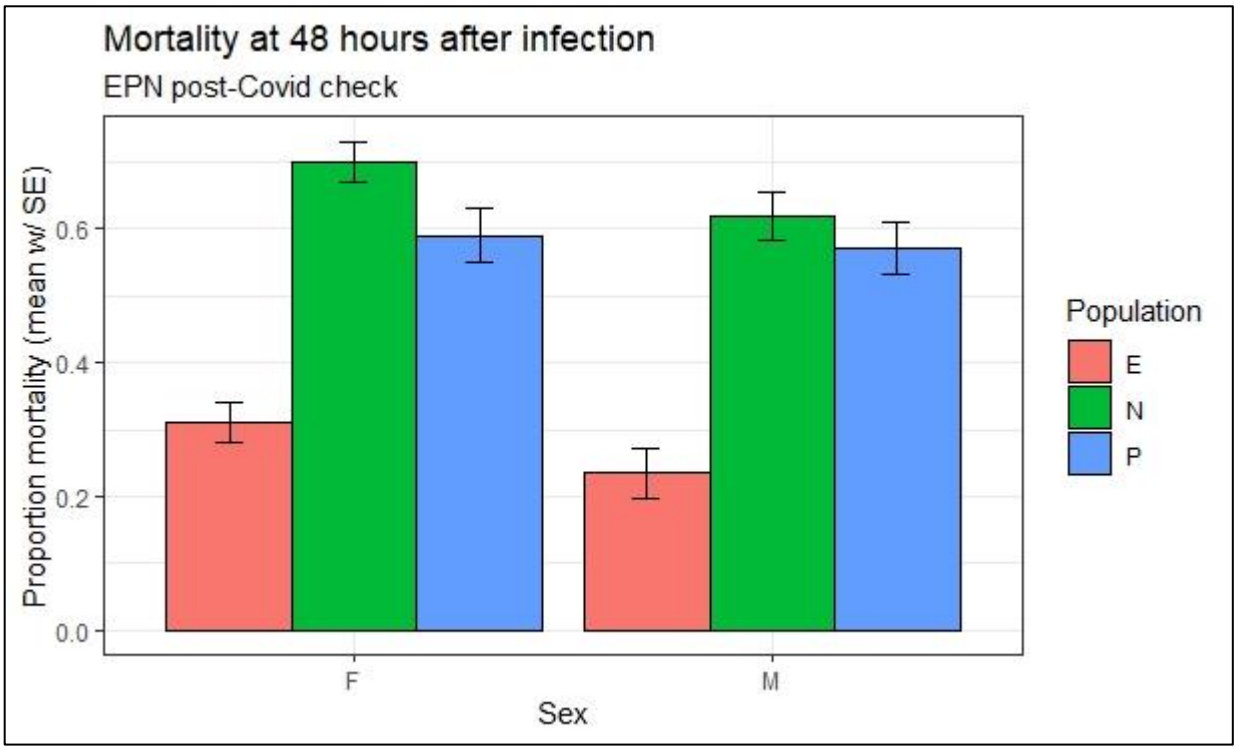


**Figure 1.2:** Mortality at 48hrs after infecting EPN flies with *E.faecalis.* On Y-axis, proportion mortality is plotted with sexes on the X-axis [44]

# MATERIALS AND METHODS

EXPERIMENTAL POPULATION: A set of replicate Drosophila melanogaster populations, selected for improved post-infection survival, were used for the experiments in this thesis. The selection lines were derived from the Blue Ridge Baseline (Gupta, V., Zeeshan, S. A. and Prasad, N. G. 2013. Sexual activity increases resistance against Pseudomonas entomophila in male Drosophila melanogaster. BMC Evolutionary Biology. 13: 185). Four baseline populations, BRB1-4, were used to derive the selection lines; these four populations share a common ancestor and had been maintained independently for ca. 150 generations by the time the selection lines were derived. The baseline populations are maintained on a 14-day discrete generation cycle, on standard banana-jaggery-yeast food medium (Table 2.1), at 25 $^{O}$C and 12:12 LD cycle.

From each baseline population (BRBi), three populations were derived (Aparajita et al (in preparation)):

a. Ei: Selected for improved post-infection survival; every generation 200 females and 200 males are infected (see below) with a Gram-positive bacterial pathogen, *Enterococcus faecalis*, and 96 hours post-infection the survivors are allowed to reproduce to start the next generation. The dosage of infection is adjusted to ensure about 50% mortality at every round of selection.
b. Pi: Control for the infection process; every generation 100 females and 100 males are sham-infected (see below), and 96 hours later survivors are allowed to reproduce. Negligible mortality happens in this population..
c. Ni: Uninfected control; every generation 100 females and 100 males are sorted under light $CO_2$ anesthesia, and 96 hours later all individuals are allowed to reproduce.

E1, P1, and N1 were derived from BRB1 and so forth, implying that populations with the same subscript share a recent common ancestor, and are part of a 'Block'. Each block is handled separately during maintenance and experimentation.

The detailed maintenance of the selected populations is described in figure 2.1. These populations are maintained on a 16-day discrete generation cycle, in the same physical environment as their ancestors.

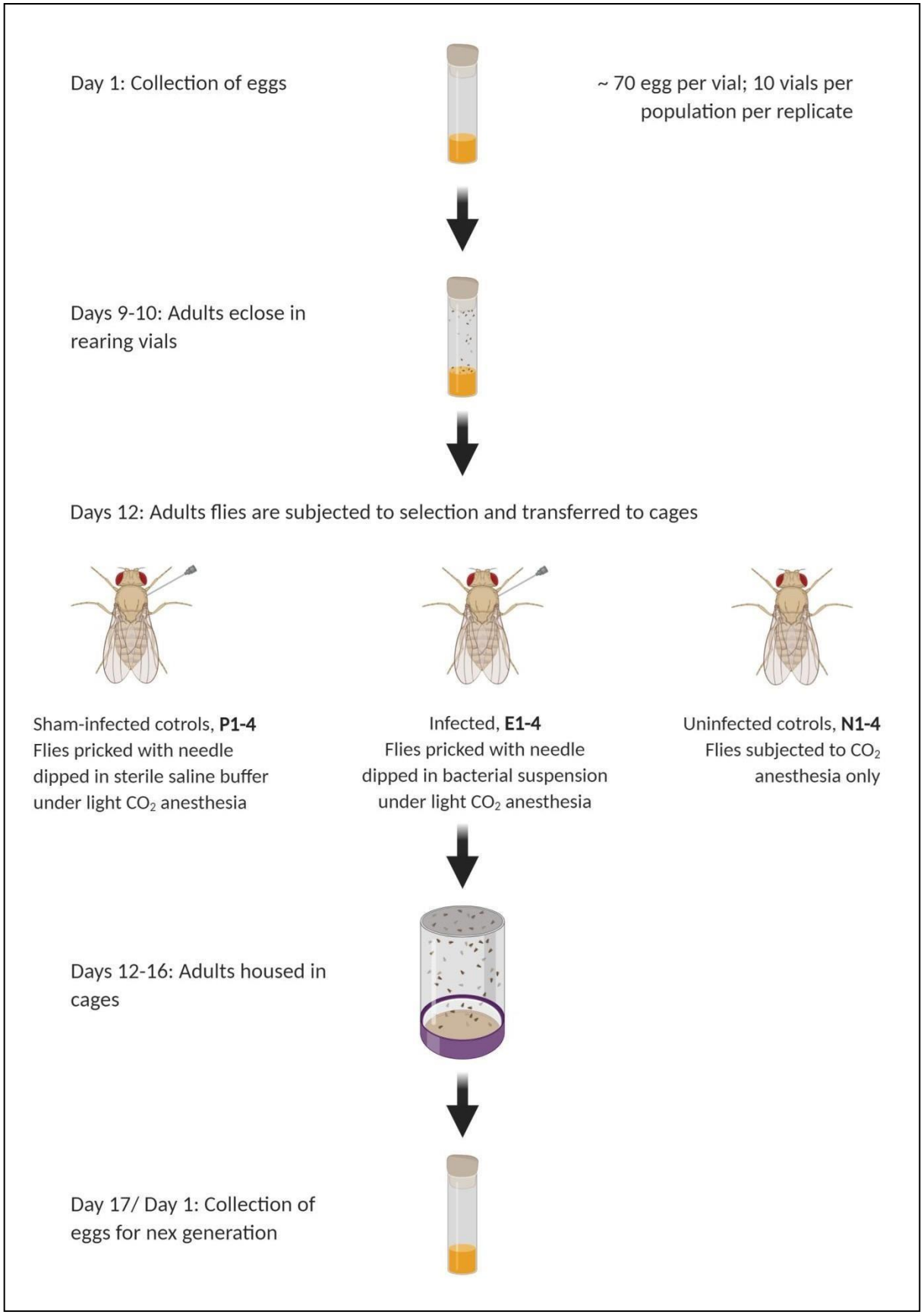


**Figure 2.1**: Maintenance of EPN population (Image made using Biorender app)

FLY FOOD COMPOSITION: Please refer to the table below for the food composition of 1litre volume of food.

| Ingredient | Amount: solids (gm), liquids (mL) |
|---|---|
| Banana | 205 |
| Barley | 25 |
| Jaggery | 35 |
| Yeast | 36 |
| Agar | 12.4 |
| Water (to mix with Agar-Jaggery) | 1000 |
| Ethanol (to mix with yeast) | 22 |
| Water | 180 |
| p-Hydroxy methyl benzoate (Preservative) | 2.4 |
| Ethanol (to mix with benzoate) | 23 |

**Table 2.1**: Fly food composition per litre volume of food

BACTERIAL STOCKS: *Enterococcus faecalis*, a Gram-positive bacterial entomopathogen, was used for both maintenance of the selection regime and for the experiments. (We are thankful to Prof. Brian P Lazzaro, Dept. of Entomology, Cornell University for lending us this strain of *E. faecalis*.) Bacteria are stored as frozen glycerol stocks and cultured on Luria Bertani (Miller) broth (HiMedia Laboratories Pvt. Ltd.) as and when necessary. For infections, a primary culture is seeded from bacterial glycerol stock and allowed to grow overnight at 37 $^{O}$C and 150 rpm (optimal growth conditions for *E. faecalis*). This culture is then diluted 2:100 to establish a secondary culture, which is grown till the log phase of growth. The bacterial cells are then pelleted down and resuspended in sterile $MgSO_4$ solution at OD = 1.5 (optical density measured at 600 nanometers). For every infection time-point (see EXPERIMENTAL DESIGN), a fresh culture was set up every 4 hours; refer to table 2.2 for the schedule.

| Time of $1^0$culture | Time of $2^0$ culture | Time of infection | ZT (lights on at 8:30) |
|---|---|---|---|
| Day 13 23:30 | Day 14 9:30 | Day 14 13:30 | 5 |
| Day 14 03:30 | 13:30 | 17:30 | 9 |
| 07:30 | 17:30 | 21:30 | 13 |
| 11:30 | 21:30 | Day 15 01:30 | 17 |
| 15:30 | Day 15 01:30 | 05:30 | 21 |
| 19:30 | 05:30 | 09:30 | 25 |
| 23:30 | 09:30 | 13:30 | 29 |

**Table 2.2**: Schedule for setting up of cultures and infections for the experiment. Same schedule was followed for both sexes.

INFECTION PROTOCOL: Flies are infected by pricking them in the thorax with a 0.1 mm Minutien pin (Fine Science Tools, CA) dipped in the bacterial suspension, under light $CO_2$ anaesthesia. For sham-infection, flies are handled similarly except that they are pricked with pins dipped in sterile $MgSO_4$. Uninfected controls are subjected only to temporary light $CO_2$ anaesthesia.

EXPERIMENTAL DESIGN: The experiments were carried out when the selected populations were between generations 69 to 79 of forward selection. Before experiments, selection was relaxed for one generation in order to avoid any parental effect ('standardization'). Eggs were collected from standardized E and N population cages at a density of 70 eggs per vial (6-8 ml of food); 40 such vials were set up for each population per block. For logistic difficulties, the P populations were excluded from this study. Virgin flies (freshly eclosed flies, within 6 hours of eclosion) were collected from these vials 10-11 days post egg-collection, and held at a density of 10 adults per vial in single sex vials. The experiment with each sex was carried out separately. 100 vials of virgin flies were collected for each population per block, and the flies were aged till day 12 in the same vials. Flies were flipped into fresh food vials on day 12 and maintained in these vials till the time of infection. The flies were continuously maintained in their natural LD cycle during the entire experiment. Infections during the night (2030 – 0830 hours) were carried under dim red-colored light.

To study the effect of time of infection on mortality, we infected sub-sets of flies every 4 hours, starting at ZT5 (1330 hours) on day 14 and finishing at ZT29 (or, ZT5, 1330 hours) on day 15. At

every time point, 8 vials worth of flies were infected and 2 vials worth of flies were sham infected for each population. Infection was carried out by the same two infectors for every time point, for all blocks, for both sexes. Vials were observed for mortality every 4-6 hours till 48 hours post-infection. Proportion mortality for each vial was calculated after 48 hours post-infection, and used for statistical analysis.

STATISTICAL ANALYSIS: Statistical analyses was performed using R (v3.6.2); 'lmerTest' package was used for mixed effect ANOVA, 'emmeans' package was used for Tukey's HSD, and 'effectsize' package was used for calculation of effect sizes [42, 43, 45]. 'Rmisc' and 'ggplot2' were used for ploting the data [44]. Proportion of flies dead ("proportion mortality") in each vial at 48 hours post-infection was used as response variable for statistical analyses, with each vial being the unit of replication. Proportion mortality was analysed using mixed-effect ANOVA (Type III Sum of Squares), and Tukey's HSD was used for pairwise comparisons. Proportion mortality was modelled as:

Proportion mortality ~ Population + ZT + Population:ZT + (1|Block) + (1|Infector),

where 'Population', 'ZT', and 'Population:ZT' were included as fixed factors, and 'Block' and 'Infector' were included as random factors.

Since the sexes were handled separately during experiments, the data from each sex was also analysed separately. Aditionally, since there was negligible mortality in sham-infected treatment, data from that treatment was excluded from

# RESULTS

Effect of time of infection (ZT) and population (E vs. N) on proportion mortality at 48 hours post-infection was tested for using ANOVA (Type III Sum of Squares).

There was a significant effect of both population ($p < 0.001$) and time of infection ($p < 0.05$) on female mortality. The interaction between population and time of infection was not significant ($p = 0.764$), indicating that both populations behaved similarly across different times of infection. Although the effect of time of infection on female mortality was significant, the effect-size estimate was too low (partial $Eta^2 = 0.05$; 95% CI: 0.01, 0.08). This suggests that time of infection doesn't have much causal role in determining female mortality. This is consistent with the observation that mortality does not fluctuate across ZTs in any specific pattern (Figure 3.1) as would be expected for a trait under circadian control.

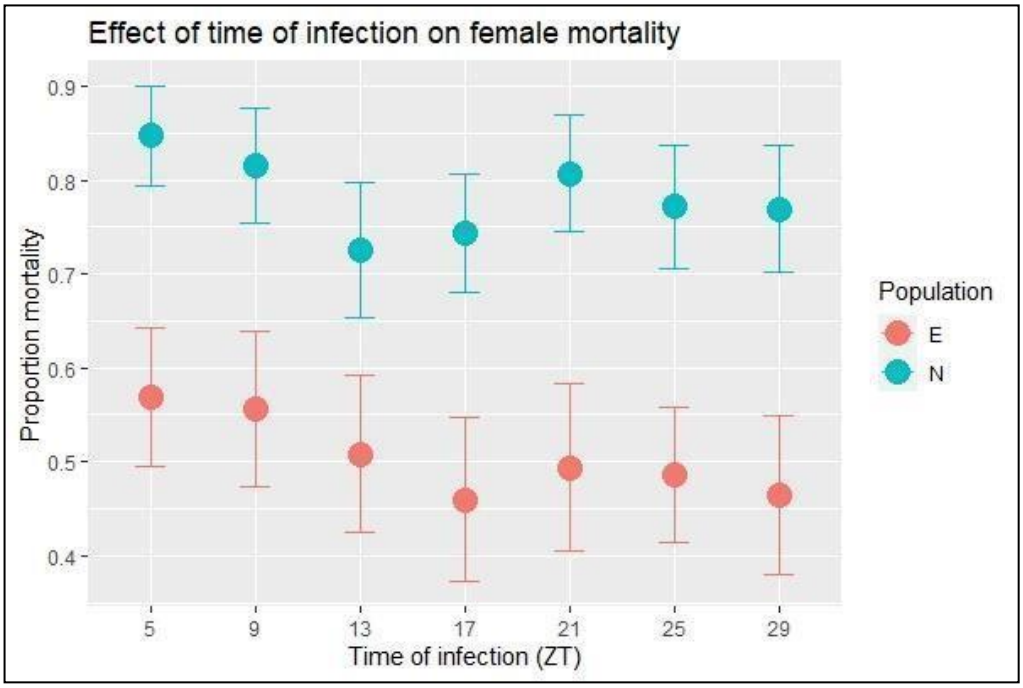


**Figure 3.1**: Effect of time of infection (ZT) on female mortality. Proportion mortality at 48 hours post-infection is plotted on Y-axis, and ZT values are plotted on X-axis. Error bars denote 95% confidence intervals around the mean.

| | Sum Square | Mean Square | Num DF | Den DF | F value | p-value | Eta$^2$ (partial) | 95% CI |
|---|---|---|---|---|---|---|---|---|
| Population | 8.617 | 8.6167 | 1 | 442.98 | 299.0 | <2e-16*** | 0.40 | [0.34, 0.46] |
| ZT | 0.603 | 0.1006 | 6 | 442.98 | 3.489 | 0.00221** | 0.05 | [0.01, 0.08] |
| Population *ZT | 0.096 | 0.0161 | 6 | 442.98 | 0.557 | 0.76407 | 7.50e-03 | [0.00, 0.02] |

**Table 3.1**: Table for Analysis of Variance (Type III Sum of Squares) and effect sizes for female mortality data.

Population was the only factor that had any significant effect on male mortality ($p < 0.001$); neither time of infection ($p = 0.084$) nor the interaction between population and time of infection ($p = 0.167$) had any significant effect. As expected the effect-size estimate was also too low for males (partial Eta$^2$ = 0.02; 95% CI: 0.00, 0.05).This suggests that time of infection is not a causal factor in determining male mortality (Figure 3.2).

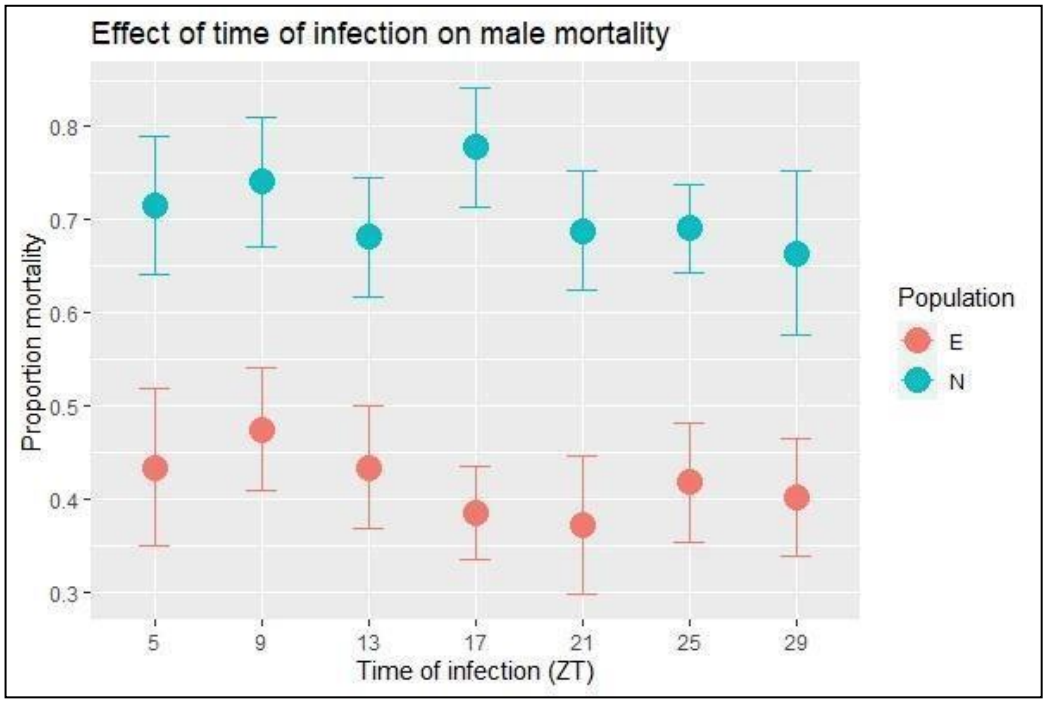


**Figure 3.2**: Effect of time of infection (ZT) on male mortality. Proportion mortality at 48 hours post-infection is plotted on Y-axis, and ZT values are plotted on X-axis. Error bars denote 95% confidence intervals around the mean

| | Sum Square | Mean Square | Num DF | Den DF | F value | p-value | Eta$^2$ (partial) | 95% CI |
|---|---|---|---|---|---|---|---|---|
| Population | 9.511 | 9.511 | 1 | 442.97 | 362.7 | <2e-16*** | 0.45 | [0.39, 0.51] |
| ZT | 0.294 | 0.0491 | 6 | 442.97 | 1.871 | 0.08424 | 0.02 | [0.00, 0.05] |
| Population *ZT | 0.240 | 0.0400 | 6 | 442.97 | 1.525 | 0.16792 | 0.02 | [0.00, 0.04] |

**Table 3.2**: Table for Analysis of Variance (Type III Sum of Squares) and effect sizes for male mortality data.

# DISCUSSION

Our results suggest that there is a significant effect of time-of-infection on survivorship when female *Drosophila melanogaster* is infected with gram positive *Enterococcus faecalis.* This is consistent with Lee and Edery, 2008 study wherein they also used virgin female flies. However, I would like to point out that as shown in table .. (table showing significance values of each pair of ZT) the circadian in females is not as robust as seen in previous studies. There is no significant difference in the mortality between flies of consecutive infection time points. Although in Stone *et.al.* 2012, it has been found that when WT males infected at ZT07 died significantly more than ZI19, no such pattern was observed in the males of our population. The reason for these discrepancies is unclear. But I speculate that it could be due one of the following reasons. The populations used in Lee and Edery, 2008 and Stone *et.al* 2012, where they showed survival rate vary as a function of time of infection, are all inbred populations like Canton S, *yw,* and Oregon R. The genetic variation in inbred lines is less as compared to outbred lines like the one used in our experiments. Inbreeding increases the probability of individuals becoming homozygous over generations. Outbred lines, on the other hand, more closely resemble the biological variation found in nature. So, it is possible that the standing genetic variation from 4 different blocks could have resulted in flies not showing a circadian pattern when pooled together. It is also possible that the flies are not in-phase at a population level. In other words, the rhythm of individual flies is not synchronized to begin with. It is known that TIM regulates resistance mechanism in flies in pathogen specific manner [39], so it is possible that *Enterococcus faecalis* does not show a time dependent variation in survivorship. Further experiments using mutants and/or more bacterial strains are necessary to know the reason behind why males and females show differences in results. It is very evident that the selection for better immune response has been successful in both males and females. Another peculiar thing that must be discussed is that we infected flies on day 14 at ZT5 and on day 15 at ZT29, which is the same time of the day i.e. 13:30 but still we observed that, in females, the mortality is significantly less on day 15 as compared to day 14. The reason behind this is unclear. We flip the flies into fresh vials on day 12, so flies infected on day 14 are in the same food for 2 days unlike the flies infected on day 15 which stay in the same food for 24hrs extra before infection. Unpublished data from lab indicates that old food

increases immunity in flies. It is also possible that immunity in flies increases with age during the middle age. Further experiments are required to resolve these confounding factors. Taken together, our results suggest that female flies are more susceptible to daytime infections than night time.

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