Supplemental Data

Circadian Regulation in the Ability of *Drosophila* to Combat Pathogenic Infections

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Supplemental Experimental Procedures

Fly Stocks

The *Canton-S*, *Oregon R*, *yw*, and clock mutant strains used in this study were descendents of stocks that were maintained in our lab for several years. *Oregon-R* flies were a gift from S. Kurata, and all the clock mutant strains used in this study are in a *yw* background; i.e., *ywper*^{01} (a gift from A. Sehgal), *yw;tim*^{01} (a gift from A. Sehgal), *yw;;cyc*^{01} (a gift from J. Hall), and *yw;;Clk*^{Jrk} (a gift from R. Allada).

P. aeruginosa Culture

The PA14 *plcs* strain used in this study was obtained from L. Rahme (Harvard Medical School) [S1]. For every experiment, a glycerol stock was freshly streaked onto an LB/gentamycin plate. After an overnight incubation, a single colony was picked and grown in 1ml of LB/gentamycin until this seed culture reached a logarithmic phase. Subsequently, the culture was diluted in 25 ml of LB/gentamycin and grown until the desired A_{600} concentration was reached (see below). Finally, the bacterial culture was centrifuged and the pellet resuspended in LB media to obtain an A_{600} reading of 100 and kept on ice during infection. Needles were directly placed in this concentrated bacterial solution and used for the infection.

S. aureus Culture

For every experiment, a glycerol stock of *S. aureus* (ATCC 10390) was freshly streaked onto an LB plate. Subsequently, a single colony was picked and grown in 2 ml of LB overnight. This seed culture was diluted in 10 ml of LB and grown until its A_{600} value reached 5.0. Finally, the bacterial culture was centrifuged and the pellet resuspended in LB media to obtain an A_{600} reading of between 10 and 50. Needles were dipped into the concentrated bacterial cultures and used to infect adult wild-type flies (*Canton-S* or *Oregon R*) at CT5 and CT17, as described for *P. aeruginosa*. Similar daily rhythms in percent survival were observed for *Canton-S* and *Oregon R* flies (data not shown), and the data from several independent experiments combined to generate the profiles shown in Figure S2B.

Survival Rates

We used female flies to avoid potential complications due to sexual dimorphism in immunity [S2, S3]. For each survival experiment, approximately 100 young adult female flies (2–4 days old) of the same genotype were placed in a bottle and entrained under 12 hr light/12 hr dark cycles (LD; where zeitgeber time 0 [ZT0] is defined as lights-on) at 25°C for 2 days. Infections were performed on either the third day of LD or second day of constant darkness (DD), as indicated in the text. For a given genotype and infection time, approximately 40–60 flies were infected with PA14 *plcs*, and 20 flies were treated as a contemporaneous mock-injury group to assess the impact of injury on survival (Table S1). To infect flies, we used standard procedures whereby individuals were anethesized with CO_2 and lightly pricked in the abdomen with a chemically sharpened tungsten needle dipped into a concentrated bacterial solution or LB media (mock injury). After infection or mock injury, flies were distributed into groups of about 20 individuals and placed in fresh vials, followed by a return to the same lighting and temperature condition in which they had been housed before treatment. Because the virulence of *P. aeruginosa* is proportional to its population density [S4], we optimized infection conditions such that the phase of the bacterial culture harvested had an A_{600} value between 3.0–3.2 for LD infections and 3.45–3.57 for DD infections. Also, in our standard conditions we delivered approximately 200–400 bacteria per fly (Figure 2). This combination of both the phase of the bacterial culture and the number of bacteria delivered resulted in a mixed response with flies that exhibited rapid death and those that survived throughout the testing period. In our experimental setting, flies that survived the first 48 hr did not die for the rest of the observation period (at least 1 week) and were considered "survivors" (Figure 1A and Figures S1, S2A, and S3B). To better compare genotypes, clock mutant and the control *yw* flies were treated together. The entire procedure of infection, collection and counting was performed under a dim red light (Kodak safelight lamp) for nighttime or DD infections.

Bacterial Growth Assay

At 0, 5, 10, and 23 hr postinfection, flies were put in ice-cold 70% ethanol and rinsed once to remove any bacteria remaining on the surface. Then, ice-cold LB media was added and flies were homogenized, individually or as a pool of 10 individuals, with a motorized pestle. Serial dilutions of each fly extract was plated onto LB/gentamycin plates and incubated at 37°C overnight. Visible colonies from the serially diluted homogenates were counted and averaged to calculate the bacterial titer in each group of flies. To attain more reliable comparisons, each experiment included control and clock mutant flies (*yw*, *per*^{01}, and *Clk*^{Jrk}) infected at CT5 and CT17 (Figure 2). For each time point and genotype, data from at least three independent experiments were pooled. For each experiment, a portion of flies representing each genotype and time of infection were not homogenized but monitored for at least one week to ensure that the survival rates were as anticipated. In rare cases where anomalous survival data were obtained (e.g., little to no mortality of infected flies throughout the week-long observation period), we did not use the corresponding results obtained from the bacterial assays.

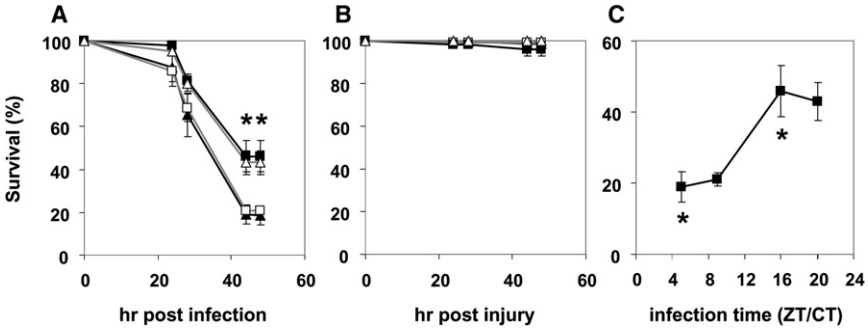

Figure S1. Time-of-Day Differences in the Survival Rates of *yw* Flies Infected with *P. aeruginosa* Are Observed over a Wide Range of Bacterial Doses

(A) Time course for the survival rates of *yw* flies infected with PA14 *plcs* at ZT5 (filled triangles, n = 235), ZT9 (open squares, n = 234), ZT16 (filled squares, n = 230), or ZT20 (open triangles, n = 233) during LD when the initial bacterial dose was 5- to 20-fold less than the standard amount used in Figures 1–4 and Figures S3 and S4 (A_{600} reading of the final bacterial solution ranged between 5 and 20). Asterisks (*) indicate significantly higher survival rates for the ZT16 and ZT20 groups compared to the ZT5 and ZT9 groups (one-way ANOVA, $p < 0.005$; Tukey-Kramer HSD, $\alpha = 0.05$). At $\alpha = 0.01$, only the ZT5 group died significantly more than did the ZT16 group. Results reflect the average of five independent experiments, one of which was performed on the first day of DD and produced similar results (data not shown). Error bars indicate SEM.

(B) Time course for the survival rates of control *yw* flies that were treated with clean needles at ZT5 (filled triangles, n = 195), ZT9 (open squares, n = 194), ZT16 (filled squares, n = 194), or ZT20 (open triangles, n = 194).

(C) Survival rates of *yw* flies infected with PA14 *plcs* at ZT5, ZT9, ZT16, or ZT20. The corresponding data at 48 hr postinfection shown in (A) was plotted to better show the daily rhythm in survival rates.

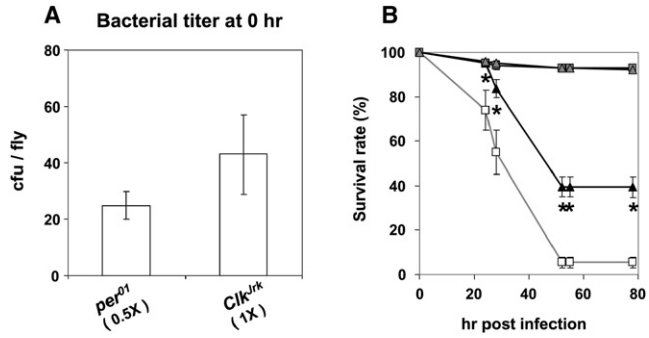

Figure S2. *Clk*^{Jrk} Flies Exhibit Higher Survival Rates Compared to *per*^{01} Flies Even When Infected with Approximately Twice the Bacterial Dose as that Used for *per*^{01} Flies

(A) The average titer of PA14 *plcs* present in *per*^{01} or *Clk*^{Jrk} flies immediately after infection. Approximately half the amount of bacteria was used to infect *per*^{01} flies (n = 30) compared to *Clk*^{Jrk} flies (n = 20). Results are the average of two independent experiments. Error bars indicate SEM.

(B) Time course for the percent survival of *per*^{01} or *Clk*^{Jrk} flies infected with PA14 *plcs* at CT5 during the second day of DD. *Clk*^{Jrk} flies (filled triangles, n = 414) have higher survival rates compared to *per*^{01} flies (open squares, n = 120) despite the higher average bacterial dose used to infect *Clk*^{Jrk} flies (two-tailed Student's t test, $p < 0.005$ at 24 hr, $p < 0.05$ at 28 hr, $p < 0.01$ after 52 hr postinfection, $\alpha = 0.05$). Mock-injury groups are also shown (gray triangles, *Clk*^{Jrk}, n = 185; gray squares, *per*^{01}, n = 161). Results are the average of nine and two independent experiments for *Clk*^{Jrk} and *per*^{01} flies, respectively. Error bars indicate SEM.

Quantitative RT-PCR

In each experiment, at least 20 infected or mock-injured flies treated at either CT5 or CT17 were frozen on dry ice at 0, 2, 5, 9, or 22 hr postinfection and kept at −80°C until ready to process. Fly heads were separated from their bodies with sieves and crushed in 200 μl of TRI Reagent (Sigma) with a motorized pestle [S5]. Total RNA was prepared according to the manufacturer's instruction, and the final RNA pellet was resuspended in 12 μl or 100 μl of DEPC-treated water for head or body extract, respectively. 5 μl of total RNA solution were used for reverse transcription (Omniscript RT, QIAGEN). The resulting cDNAs were diluted 10-fold in 10 μM of Tris (pH 8.0). Subsequently, 2 μl of cDNA solution was added to a total of 30 μl and PCR reactions performed in triplicate (QuantiTect SYBR Green PCR, QIAGEN). Real-time PCR was performed in a 96-well plate on an ABI prism (Applied Biosystems) with the following conditions: a cycle of 15 min at 95°C to activate HotStarTaq DNA polymerase, followed by 40 cycles of 15 s at 94°C, 30 s at 60°C, and 30 s at 72°C. The standard curve was generated for every run and the absolute copy number of the gene of interest was calculated based on Ct (threshold cycle) values. Finally, the values were normalized to the copy number of the *rp49* gene. The efficiency of all the PCR reactions was at least 90%. The sequences of the PCR primers used in this study are provided in Table S5). As above for the bacterial growth assays, a portion of flies representing each genotype and time of infection were monitored for at least one week to ensure that the survival rates were as anticipated and results from experiments with anomalous survival data were not used. For each genotype, the values for RNA levels obtained at the same postinfection collection time were compared for the CT5 and CT17 groups.

Statistical Analysis

Survival rates of flies infected at different times of a day were evaluated by one-way ANOVA to determine if they showed statistically significant differences as a function of infection time. Comparison between all pairs of groups was performed by Tukey-Kramer HSD analysis to determine the lowest and highest survival rates in cases in which the survival rates varied among groups. Power analysis was also done to assess the adequateness of the sample size (Figures 1B–1F and Tables S2 and S3). For pairwise comparisons between CT5 and CT17 groups for a given genotype or between two genotypes, two-tailed Student's t test was used. All the statistical analysis described above was carried out with JMP6 software (SAS).

Supplemental References

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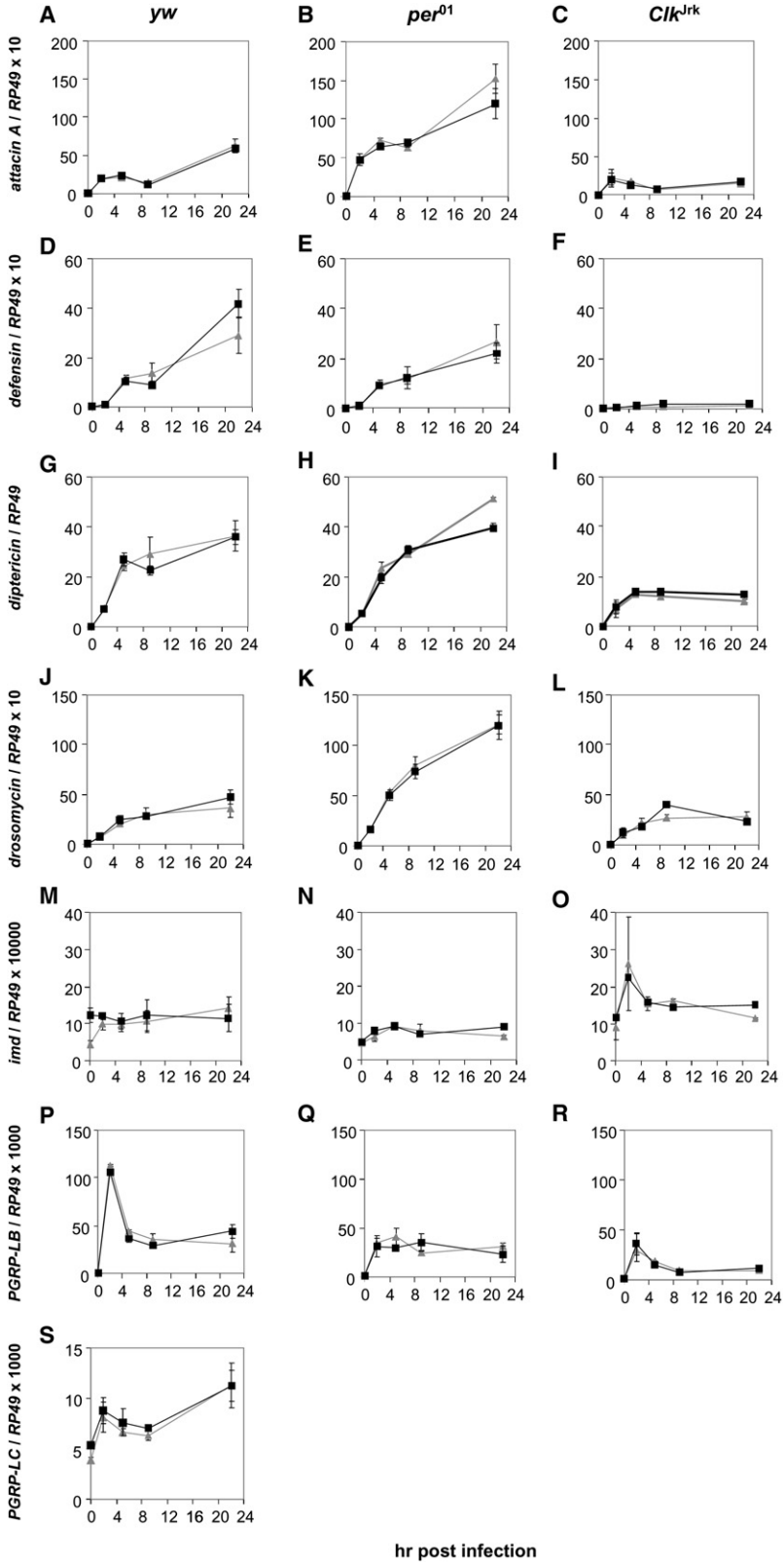

Figure S3. No Time-of-Day Effects on the Induction Kinetics for *attacin A*, *defensin*, *diptericin*, *drosomycin*, *imd*, *PGRP-LC*, and *PGRP-LB*

(A–S) Control *yw* and clock mutant flies (as indicated) were infected at either CT5 (gray triangles) or CT17 (black squares), collected at the indicated times postinfection, and mRNA levels were measured via real-time RT-PCR. For each gene, none of the RNA values measured at a particular time postinfection showed statistically significant differences between the CT5 and CT17 groups (two-tailed Student's t test, $p > 0.05$). Results from at least three independent experiments were averaged, except that *Clk*^{Jrk} data were derived from two experiments. Error bars indicate SEM.

Table S1. Percent Survival of Mock-Injury Groups

Cycle	Genotype	n	Infection time (ZT/CT)	Survival (%) (Mean ± SEM)	Independent Experiments
LD3	*yw*	125	1	96.0 ± 0.0	5
LD3	*yw*	125	5	100.0 ± 0.0	5
LD3	*yw*	125	9	97.3 ± 1.3	5
LD3	*yw*	125	13	98.7 ± 1.3	5
LD3	*yw*	125	17	100.0 ± 0.0	5
LD3	*yw*	125	21	97.3 ± 1.3	5
DD2	*yw*	150	1	97.5 ± 1.2	6
DD2	*yw*	150	5	97.0 ± 1.9	6
DD2	*yw*	150	9	98.5 ± 1.0	6
DD2	*yw*	149	13	98.0 ± 0.9	6
DD2	*yw*	150	17	96.7 ± 1.1	6
DD2	*yw*	150	21	96.7 ± 1.2	6
DD2	per^{01}	69	1	89.3 ± 7.1	3
DD2	per^{01}	70	5	93.0 ± 2.5	3
DD2	per^{01}	70	9	95.7 ± 0.3	3
DD2	per^{01}	69	13	90.0 ± 5.8	3
DD2	per^{01}	70	17	79.3 ± 8.4	3
DD2	per^{01}	70	21	81.0 ± 10.7	3
DD2	tim^{01}	70	1	91.3 ± 4.7	3
DD2	tim^{01}	70	5	94.3 ± 1.2	3
DD2	tim^{01}	70	9	97.3 ± 2.7	3
DD2	tim^{01}	70	13	94.7 ± 1.3	3
DD2	tim^{01}	69	17	95.7 ± 2.3	3
DD2	tim^{01}	70	21	92.0 ± 4.6	3
DD2	cyc^{01}	75	1	93.3 ± 1.3	3
DD2	cyc^{01}	75	5	98.7 ± 1.3	3
DD2	cyc^{01}	75	9	100.0 ± 0.0	3
DD2	cyc^{01}	75	13	97.3 ± 1.3	3
DD2	cyc^{01}	75	17	98.7 ± 1.3	3
DD2	cyc^{01}	75	21	98.7 ± 1.3	3
DD2	Clk^{Jrk}	65	1	95.3 ± 0.3	3
DD2	Clk^{Jrk}	65	5	90.7 ± 0.7	3
DD2	Clk^{Jrk}	65	9	93.7 ± 4.5	3
DD2	Clk^{Jrk}	65	13	92.3 ± 4.3	3
DD2	Clk^{Jrk}	65	17	90.7 ± 5.8	3
DD2	Clk^{Jrk}	65	21	86.7 ± 10.9	3

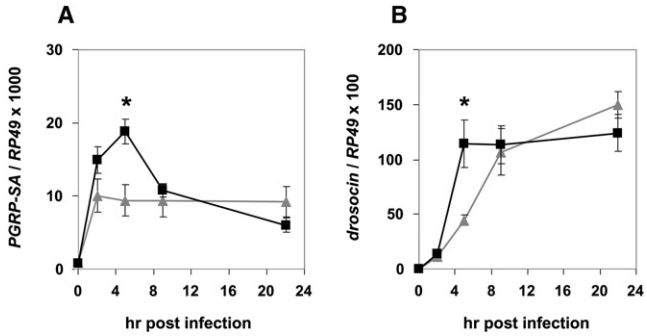

Figure S4. Time-of-Day Effects on the Postinfection Profiles for *PGRP-SA* and *drosocin* Also Are Observed in the Body

Control *yw* flies were infected at either CT5 (gray triangles) or CT17 (black squares), collected at the indicated times, and RNA levels measured. Asterisks (*) indicate significantly higher mRNA levels for the *yw* CT17 group compared to its CT5 group (two-tailed Student's t test, $p < 0.01$). Results reflect the average of four independent experiments. Error bars indicate SEM.

Table S2. Power Analysis of the Survival Rates for *yw* and Clock Mutant Flies Infected in DD

Genotype	n	Independent Experiments	p Value of One-Way ANOVA	Power[a]	LSN[b]	Number of Observations	LSE[c]
yw	321	6	0.0029	0.9460	22	36	4
per^{01}	155–160	3	0.9739	0.0741	259	18	44
tim^{01}	137–140	3	0.8825	0.1051	123	18	21
cyc^{01}	147–151	3	0.4178	0.2621	41	18	7
Clk^{Jrk}	125–139	3	0.6614	0.1690	65	18	11

[a] Power represents the probability to obtain significance at or below a given p value for a given situation.

[b] LSN (least significant number) represents the total number of observations that would give rise to a specified p value (0.05 in this power analysis) given that the data has the same form.

[c] LSE (least significant experiment) represents the total number of experiments that would give rise to a specified p value (0.05 in this power analysis) given that the data has the same form. Each experiment involves six observations (survival rates of flies infected at six different times; see Figure 1).

Table S3. Power Analysis of the Survival Rates of *per*^{01} and *Clk*^{Jrk} Flies Infected at CT5 or CT17

Genotype	n	Independent Experiments	p Value of Two-Tailed Student's t Test	Power	LSN	Number of Observations	LSE[a]
per^{01}	366	8	0.6593	0.0705	306	16	153
Clk^{Jrk}	315	7	0.6071	0.0776	196	14	98

[a] LSE (Least Significant Experiment) represents the total number of experiments that would give rise to a specified p value (0.05 in this power analysis) given that the data has the same form. Each experiment involves two observations in this analysis (survival rates of flies infected at CT5 or CT17; see Figure S3B).

Table S4. Two-Tailed Student's t Tests from Results Shown in Figure 3

Collection Time (hrs Postinfection.)	Groups to Compare	p Value
10	*yw* CT5 versus *yw* CT17	0.0282
10	*per*^{01} CT5 versus *per*^{01} CT17	0.4898
10	*Clk*^{Jrk} CT5 versus *Clk*^{Jrk} CT17	0.7474
10	*per*^{01} (CT5 + CT17) versus *Clk*^{Jrk} (CT5 + CT17)	0.0022
23	*yw* CT5 versus *yw* CT17	0.0107
23	*per*^{01} CT5 versus *per*^{01} CT17	0.6257
23	*Clk*^{Jrk} CT5 versus *Clk*^{Jrk} CT17	0.5783
23	*per*^{01} (CT5 + CT17) versus *Clk*^{Jrk} (CT5 + CT17)	0.0439

Table S5. Sequences of the Primers Used for Quantitative RT-PCR

Gene	Forward Primer (5′–3′)	Reverse primer (5′–3′)
attacin A	TCCTTGACGCACAGCAACTTC	GGCGATGACCAGAGATTAGCAC
defensin	TCTCGTGGCTATCGCTTTTGC	CCACATCGGAAACTGGCTGA
diptericin	GACGCCACGAGATTGGACTG	CCCACTTTCCAGCTCGGTTC
drosocin	TGTCCACCACTCCAAGCACAA	CATGGCAAAAACGCAAGCAA
drosomycin	TCCTGATGCTGGTGGTCCTG	TCCCTCCTCCTTGCACACAC
imd	CCGAGCAATGTGAGTTGATTTTCG	CGTGCGTTCTGCCTTCCAATAG
PGRP-LB	CGGCGATGGCATGATTTACA	CGGCAGTTCGGTTCTCCAAT
PGRP-LC	CCTACCCGCCCAACAGTTC	GTGGTACTGCCGCCTCACCT
PGRP-SA	CGGATCTCCTTGGATTATGG	TAGTGGAGTCCCAACGAAGG
rp49	CCCACCGGATTCAAGAAGTTCC	TCGACAATCTCCTTGCGCTTC