Behavioral Effects of *amontillado* RNAi Knockdown in the *Drosophila melanogaster* Circadian Clock Neuron Network

By

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Abstract

Circadian rhythms are endogenous biological oscillations that are present in microbes, plants, nematodes, and mammals. All animals have an oscillation of approximately 24 hours, though the precise period of these oscillations varies between species. In *Drosophila melanogaster,* circadian rhythms are coordinated by the circadian clock neuron network (CCNN), which is divided into different classes of clock neurons. Many neuropeptides are synthesized in the CCNN, only some of which have been characterized. Before neuropeptides can become bioactive, their precursors have to go through neuropeptide processing. In *Drosophila* the enzyme involved in the first step of neuropeptide processing is the *Drosophila* protein convertase 2 (dPC2), also known as "*amontillado*." I have used the GAL4/UAS-RNAi genetic tool to knock down *amontillado* expression in different groups of neurons in the CCNN to observe the effects on the fruit fly's circadian rhythm of sleep and activity. Changes in such circadian rhythms in *amontillado* knockdown flies would reveal the roles that neuropeptides released from subsets of neurons play in their control.

Introduction:

I. The Circadian Cycle

Drosophila melanogaster, also known as the fruit fly, provides great opportunities as a model organism for scientific research. Not only does the fruit fly share molecular and cellular features with mammals, it is an easily reared and genetically tractable model organism, with a short generation time, and provides many offspring to work with. Circadian rhythms are endogenous biological oscillations that are present in microbes, plants, nematodes, and mammals (Herzog 2007). All animals have an oscillation of approximately 24 hours, though the precise free running period of these oscillations varies slightly between species (Herzog 2007). These circadian rhythms are synchronized by zeitgebers, which are external environmental cues like light and temperature cycles (Herzog 2007).

In mammals, circadian rhythms are driven by a "master clock," located in the suprachiasmic nuclei (SCN) of the hypothalamus. The master clock coordinates and maintains the daily behavioral rhythms of animals (Herzog 2007). Some examples of these daily behavioral rhythms include when an animal goes to sleep, when it wakes up, when it eats, and time of day when it secretes certain hormones (Herzog 2007).

The SCN of the laboratory mouse consists of approximately 20,000 clock neurons (Welsh et al. 2009). In the fruit fly, the master clock, also known as the circadian clock neuron network (CCNN), consists of approximately 150 clock neurons; 75 clock neurons per hemisphere (Shafer et al. 2006). These neurons can be divided into classes of clock neurons, which include: small ventrolateral neurons (s-LNv), large

ventrolateral neurons (l-LNv), dorsal lateral neurons (LNd), dorsal neurons 1 (DN1), dorsal neurons 2 (DN2), dorsal neurons 3 (DN3), 5th small ventrolateral neuron (5th-sLNv), and lateral posterior neurons (LPNs) (Ewer et al., 1992; Frisch et al., 1994; Figure 1). When comparing the mouse to the fly, the fly has a fewer amount of clock neurons to work with but have complex behaviors we can learn a lot from, for example, locomotor activity, sleep, and feeding.

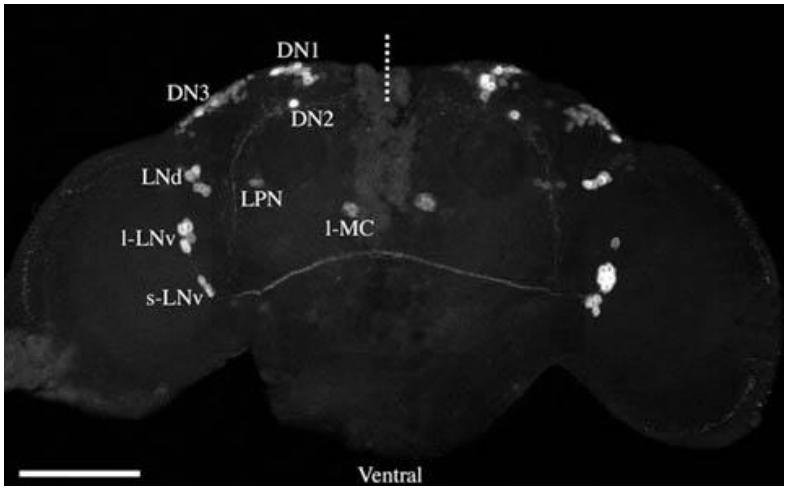

Figure 1. The Circadian Clock Neuron Network (CCNN) of *Drosophila.* The ~150 neurons are divisible into different classes of clock neurons. The various classes are indicated in the left hemisphere (Shafer et al. 2006). **s-LNv –** small ventrolateral neurons, **l-LNv** – large ventrolateral neurons, **LNd** – dorsolateral neurons, **DN-** dorsal neurons, **LPN-**lateral posterior neuron, **l-MC-**large medial cell.

Circadian clocks have an endogenous period of approximately 24 hours and synchronize with the daily light-dark cycle (Herzog 2007). Wild-type flies are most active during the dawn and evening, and they are the least active during midday and midnight (Rhea et al. 1999). To determine a free running period of an animal, it is important for an animal to be entrained in a light dark cycle and then switched into a complete darkness cycle. Entrainment is the synchronization to an external rhythm. For example, flies placed in a light/dark cycle anticipate lights on and lights off and

display consistent phases of activity and rest relative to lights-on and lights-off (Rhea et al. 1999). For the purposes of my project, this was done by placing fruit flies in 12:12 hr light/dark (LD) cycle in an incubator at 25°C for one week. To determine if a circadian rhythm persists in the absence of an LD cycle, flies were switched to constant darkness and temperature (DD) for two weeks. Under complete darkness, rhythmic behavior persists in wild-type flies and the free running period is slightly different than 24 hours (Rhea et al.1999).

The molecular circadian clock of the fly consists of a negative feedback loop involving the proteins CLOCK, CYCLE, TIMELESS, and PERIOD, and is similar to that of mammals (Herzog 2007). In the absence of TIM, PER degradation occurs quickly (Kreitzman and Foster 2005). TIM degradation occurs in the presence of light (Kreitzman and Foster 2005). Thus, light controls the phase of TIM and PER protein build up and degradation. CLOCK and CYCLE dimerize, which leads to an increase in transcription of the *period* gene (Herzog 2007). Period mRNA is then translated into PER proteins. When there is an increased level of PER protein, PER associates with TIM and inhibits CLOCK and CYCLE induced transcription of PER. When PER protein levels decrease, CLOCK and CYCLE once again produce an increase in *period* transcription (Herzog 2007).

II. Neuropeptides and Neuropeptide Processing

Another important component of circadian rhythms is neuropeptide signaling. Neuropeptides are responsible for communication between neurons and are essential for proper regulation of behavior and physiological and developmental processes

(Wegener et al. 2011). Neuropeptides, also known as peptide hormones, are synthesized by the secretory neurons of the central nervous system (CNS) (Wegener et al. 2011).

Studies of neuropeptides in the mouse SCN have led to the discovery of vasoactive intestine polypeptide (VIP), calretinin, neurotensin (NT), and gastrin releasing peptide (GRP) expression (Welsh et al. 2009). VIP is an important neuropeptide that is rhythmically released from the core of the SCN (Welsh et al. 2009). VIP has also been shown to induce the expression of *Per1* and *Per2,* which are components of the molecular clock (Welsh et al. 2009). Mice with a VIP mutation have low levels of *Per1* and *Per2*, and weak and fast-running behavioral rhythms (Welsh et al. 2009).

Similarly to the mouse, the *Drosophila* CCNN also expresses an important neuropeptide for circadian rhythms, pigment dispersing factor (PDF). In a study conducted by Renn et al. (1999), *pdf* null mutants displayed weak and fast running circadian rhythms reminiscent of the effects of loss of VIP in mouse rhythms. In *Drosophila*, other neuropeptides have been characterized within the CCNN. These include neuropeptide F (NPF), short neuropeptide F (sNPF), ion transport peptide (ITP), IPNamide (Shafer et al. 2006) and Diuretic Hormone 31 (DH31) (Renn et al. 1999; Johard et al. 2009). The existence of other un-identified neuropeptides within the CCNN is highly probable and these are likely to play important roles in circadian rhythms.

Before neuropeptides can become active for neuropeptide signaling, their precursors have to go through neuropeptide processing. Neuropeptide precursors are enzymatically cleaved and modified by various enzymes. Some of these enzymes include amontillado (amon; dPC2), carboxypeptidase D (silver), and amidating enzymes (PAL & PHM). Amontillado catalyzes the first step in peptide processing (Wegener et al. 2011).

III. What is the Question?

The central question of this thesis is "What are the effects of preventing specific groups of clock neurons from releasing neuropeptides on the behavioral circadian rhythms of the fly?" The plan of action was to block neuropeptide processing in specific clock neurons of the CCNN, through the RNAi mediated silencing of *amontillado* expression, in order to understand the specific contributions that neuropeptide signaling from clock neurons makes to circadian rhythms within the fly brain.

IV. The *D. melanogaster* Proprotein Convertase *amontillado*

Neuropeptides are enzymatically cleaved from bigger inactive peptide precursors by enzymes called protein convertases (Rhea et al. 2010). For my project, I observe and analyze the behavior of flies with an attempted knockdown of the *amontillado* gene. a*montillado* is also known as dPC2, which stands for *Drosophila* prohormone convertase 2 (Rhea et al., 2010). This dPC2 is a homolog of the human PC2 enzyme, and it ubiquitously found throughout the organism (Rhea et al. 2010). *amontillado* is responsible for the first step in neuropeptide processing, which is required for bioactive neuropeptide signaling (Wegner et al. 2011; Rhea et al. 2010). Preventing

neuropeptide processing in *D. melanogaster* is relatively straightforward thanks to powerful genetics tools like the GAL4/UAS system and RNAi mediated gene knockdown methods. Flies that are mutant for *amontillado* do not survive past early development, most of these mutant fail to hatch as first instar due to defects in molting behavior (Rhea et al. 2010). Thus, *amontillado* deficiency in the whole fly is lethal. The goal of this project is to use the GAL4/UAS system to drive *amontillado* RNAi knock down in regions of the CCNN and observe the effects on circadian behavior.

V. The GAL4/UAS System

The GAL4/UAS system is a powerful genetic tool wherein the yeast transcription factor GAL4 activates the expression of transgenes downstream of the Upstream Activation Sequence (UAS) in a tissue or cell specific manner (Figure 2). In this system fly enhancer sequences drive GAL4 expression within specific cells or tissues (Phelps and Brand 1998). Thus, flies with the GAL4 component will have GAL4 expression determined by the enhancer sequence that was used. Flies with a UAS-target gene component do not express this element anywhere in the fly in the absence of GAL4 expression.

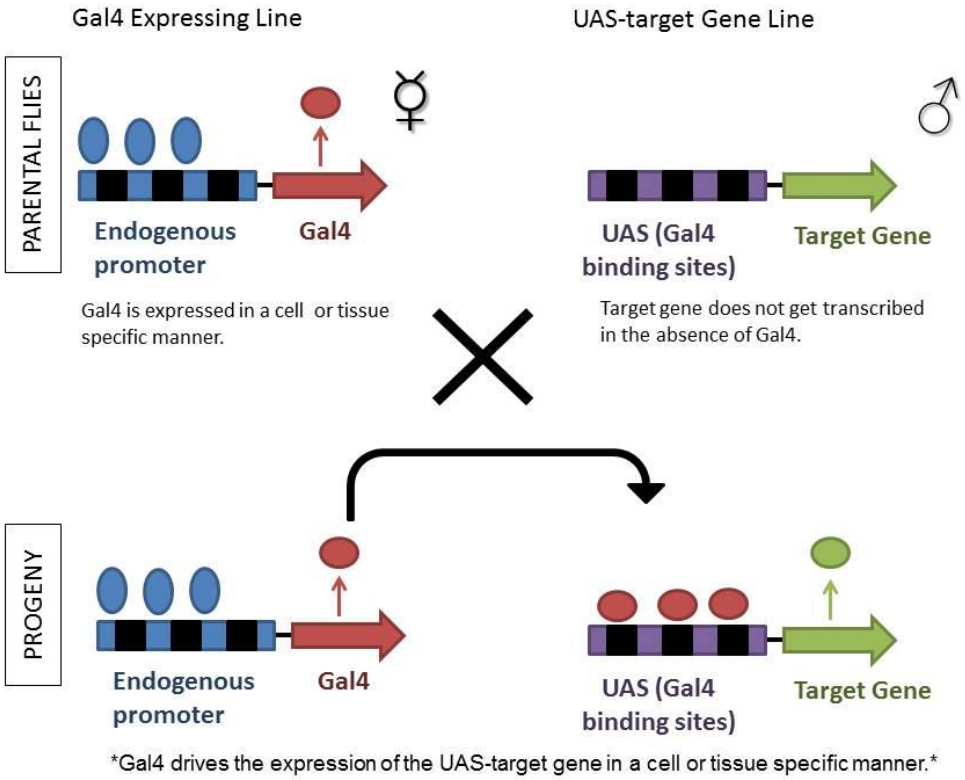

Figure 2: The GAL4/UAS System. The illustration depicts the GAL4/UAS system where GAL4 expression occurs via an endogenous cell or tissue specific promoter GAL4. When the GAL4 and UAS parental lines are crossed, GAL4 will bind the Upstream Activation Sequence (UAS); GAL4 binding sites, which control the expression of the target gene (Redrawn from Phelps and Brand, 1998).

VI. Previous Research on *amontillado*

In a study conducted by Rhea et al. (2010), the dPC2 *amontillado* was knocked down in the corpora cardiaca endocrine cells that produce the glucose regulatory peptide hormone, adipokinetic hormone (AKH). This knockdown was done using the GAL4/UAS-RNAi system. This study provided evidence that *amontillado* is required in the corpora cardiaca for the production of adipokinetic hormone peptide (Rhea et al. 2010). This hormone is responsible for increasing sugar levels from energy stores similar to glucagon's function in vertebrates (Rhea et al. 2010). Results of the study

showed a reduction in hemolymph sugar level in *Drosophila* amontillado mutants, thus establishing that *amontillado* expression had been knocked down and that AKH had failed to be processed.

Research conducted by Wegener et al. (2011) showed that a loss of *amontillado* function leads to the impairment of neuropeptide production in *Drosophila* and that *amontillado* is expressed by peptide hormone-producing neurons (Wegener et al. 2011). Larvae brain with the components amon-GAL4>UAS-GFP were immunostained with antibodies against peptides that are released by the ring gland (Wegener et al. 2011). The ring gland neuropeptides Corazonin, CC-LP2, and sNPF all colocalized with *amontillado* (Wegener et al. 2011).

Materials and Methods

I. Lethality Assay

To determine which *amontillado* UAS-RNAi lines were effective at knocking down *amontillado*, an actin-GAL4 line was used to drive *amontillado* RNAi expression throughout the fly. The actin-GAL4 line used was *y*^{1}*w*; P{Act5C-GAL4}25FO1/(CyO), y+;* + (Ito et al. 1997), and female virgin flies were crossed to males of four different UAS-amon-RNAi lines. The four UAS-amon-RNAi lines that were used are as follows: *y1v1; +; P{TRiP.HM05071}attP2* (Perkins et al. 2009), *w*: +; P{UAS-amon-RNAi}78b* (Cook 2009), *w*; P{UAS-amon-RNAi}69h* (Cook 2009), and *w*; UAS-amon-RNAi*^{28b}*;*+ (Christian Wegener, University of Würzburg; Würzburg, Germany; Rhea et al. 2010).

amontillado Drosophila mutants are not able to hatch from the egg shell leading to a lethal phenotype in *amontillado* deficient progeny (Siekhaus and Fuller, 1999). To

determine which of UAS-amon-RNAi are able to knock down *amontillado* expression in flies, I carried out four crosses with the actin-GAL4. If a UAS-amon RNAi line is successful in knocking down *amontillado* expression, no surviving offspring of this cross should contain the actin-GAL4 element. As a result, all offspring would inherit the CyO balancer and therefore have curly wings. The wing phenotypes of the progeny of all four actin- GAL4 UAS-amontillado-RNAi crosses were observed and recorded. If the *amontillado* expression knock down occurs, a straight wing phenotype would not be expected. The presence of straight winged progeny would indicate that *amontillado* expression was not knocked down. The proportion of flies of each phenotype for all four crosses was recorded and analyzed via Chi Square.

II. GAL4/UAS-RNAi Assay

Males from the UAS-amon-RNAi lines described above were crossed to virgin females of *w; clk(-856[8.2/2])-GAL4;*+ (Gummadova et al. 2009). In a later experiment, virgin female flies of genotypes (1.) *UAS-Dicer2; +; cry(13)-GAL4* (Baumgardt 2007 ; Emery 2000) and (2.) *UAS-Dicer2; +; Tim(UAS)-GAL4* (Dietzl 2007; Emery 1998) were crossed to male flies of *UAS-amon-RNAi^{28b};*+ (Rhea et al. 2010). Male progeny from all crosses ranging from 1-4 days were used for locomotor/sleep behavior assays.

III. Behavior: Locomotor Activity

Progeny of crosses were assayed for diurnal and circadian locomotor activity. Individual flies were placed in 5 mm Pyrex Glass monitor tubes (TriKinetics Inc. Waltham, MA), which were inserted into DAM2 Drosophila Activity Monitors (TriKinetics

Inc. Waltham, MA; Hermann et al. 2014). The TriKinetics system was used to record the total crosses in 5 minute bins. Male flies were used for locomotor activity behavior.

Flies were entrained to a 12 hour light and 12 hour dark (LD) cycle in an incubator held at constant temperature of 25°C for one week (Chiu et al. 2010). To determine the flies' free running period, the flies were switched to complete darkness (DD) at 25°C for 2 weeks after entrainment (Chiu et al. 2010). To determine the percent of flies displaying significant free-running periodicity, I used a chi-square periodogram analysis using Clock Lab (ActiMetrics, Wilmette, IL). The free running periods (tau) were determined for each rhythmic fly and average tau was determined for each genotype tested. Flies that failed to show a single peak above significance were considered arrhythmic.

IV. Behavior: Sleep

One-minute counts of activity were recorded throughout the length of the experiment for sleep analysis. Fruit flies were entrained for a total of 6 days to 12:12 LD and then were left in constant darkness for 9 days at 25°C. Sleep events were identified as bouts of inactivity greater than or equal to five minutes (Pfeiffenberger et al. 2010).

V. Software/Graph Generation

I used the DAMFileScan110 (TriKinetics Inc., Waltham, MA) application to sort raw data of *Drosophila* activity into 30 minute bins. Eduction plots were generated using Microsoft Excel (Microsoft Corporation, Redmond, WA). The activity of all flies of a given genotype was averaged for every 30-minute bins of activity. The data were then normalized over the 5 days of entrainment in a 12L:12D light cycle at 25°C.

To look at individual flies' activity, double plotted actograms were generated using Clock Lab (ActiMetrics, Wilmette, IL) application in MatLab (Mathworks, Natick, MA). These actograms display two consecutive days of locomotor activity on each line. Sleep data was analyzed using Counting Macro software from the Allada Lab at Northwestern University (Evanston, IL; Meissner et al. 2011). The data gathered from Counting Macro was then used in GradPad Prism 6 (Grad Pad Software Inc., La Jolla, CA) to create sleep output graphs depicting minutes of sleep per hour.

Results

I. Lethality Assay

Flies with *amontillado* deficiency are not able to hatch from the egg and die during early development (Siekhaus and Fuller, 1999). To determine which *amontillado* UAS-RNAi lines were effective at knocking down *amontillado* expression, a lethality assay was conducted. An actin-GAL4 line, with a curly wing chromosome balancer (CyO), was crossed to 4 UAS-amon-RNAi lines (Table 1). If RNAi knock down of *amontillado* expression is successful, no progeny of the cross should contain the actin-GAL4 element. Thus, the expected phenotype would only be curly wing flies because flies would inherit the CyO balancer. Straight wing flies would not be observed because *amontillado* deficiency is lethal. The proportion of flies of each phenotype for all four crosses was recorded and analyzed via Chi Square (Table 1). Three of the four *amontillado* UAS-RNAi lines were able to knock down *amontillado* as determined from the high proportion of curly winged flies on Table 1. actin-GAL4 flies that were crossed to *y1v1; +; P{TRiP.HM05071}attP2* flies had a high number of both curly (N=89) and

straight winged (N=105) flies (Table 1). To decide whether there was a significant difference between the observed phenotypes of each cross and the expected proportions of curly and straight-winged flies, a Chi Square value was calculated using the Chi Square analysis. The values for the Chi Square analysis are shown in Table 1. Through Chi Square analysis, it was concluded that the cross between actin-GAL4 and *y1v1; +; P{TRiP.HM05071}attP2* flies did not have a significant difference from the expected 1:1 ratio of wing phenotypes. We concluded was that there is no significant difference between the observed and expected phenotypes; indicating that *amontillado* expression was not successfully knocked down. The Chi Square value for all other UAS-amon-RNAi lines was higher than the probability level of 3.841 (Two-tailed P-value<0.0001). Thus, there was a significant difference between the observed and expected phenotypes of these crosses, indicating that *amontillado* had been knocked down.

II. Knocking Down *amontillado* in *clk* Expressing Cells

To first compare the locomotor activity of flies with and without functional circadian clocks we observed the lomomotor activity of wild-type, w^{1118}, flies and flies with a loss of function *per* mutation, *yw,* per^{01}. The patterns of locomotor activity for these two flies are illustrated in Figure 3.1 (A-B). Figure 3.1 (A) depicts an eduction plot for w^{1118} with arrows showing anticipation into the morning peak (lights on, ZT 0) and anticipation into the evening peak (lights off, ZT 12). This is normal behavior of wild-type flies that are placed in an LD cycle for entrainment (Konopka and Benzer 1971). Figure 3.1 (B) depicts an eduction plot for *yw, per01,* which has no apparent anticipation into the morning or evening peak.

In an attempt to knock down amontillado expression in the CCNN, we crossed *w; clk(-856[8.2/2])-GAL4;+* virgin female flies with *y1v1; +;P{TRiP.HM050713}attP2* male flies. This clk-GAL4 is expressed in all clock neuron classes (Gummadova et al. 2009). Figure 3.1 (C) shows an eduction plot for control flies with only the clk-GAL4 element. This GAL4 line displayed normal morning and evening anticipation. Figure 3.1 (D) shows an eduction plot for w^{1118}*;+;P{TRiP.HM050713}attP2/+* flies, which have only one copy of *amontillado* UAS-RNAi on the second chromosome. This fly likewise displayed morning and evening peaks of activity. Figure 3.1 (G) illustrates an eduction plot for the experimental *w;clk(-856[8.2/2])-GAL4/+;P{TRiP.HM050713}attP2/+* flies. These flies have both the GAL4 and UAS elements, and are therefore expected to express amontillado RNAi constructs throughout the CCNN. Nevertheless, these flies displayed normal morning and evening anticipation. There was therefore no apparent effect of RNAi construct expression on behavior under LD by using this UAS-amon-RNAi line. Similar negative results were seen for two other UAS-amontillado RNAi constructs (Figure 3.1, E-I).

Figure 3.2 illustrates double plotted actograms of individual flies entrained for 5 days in a 12:12 hr LD cycle, and switched to 12:12 hr DD for constant darkness. Figure 3.2 (A) depicts a double plotted actogram for a normal wild-type fly, w^{1118}. The fly maintained its rhythmicity after being switched to constant darkness, which previous studies have shown (Konopka and Benzer 1971). An actogram for the loss of function *yw,* per^{01} mutant is shown in Figure 3.2 (B). As previously shown, period mutants are arrhythmic throughout the length of the experiment under constant conditions (Konopka and Benzer 1971). The clk-GAL4 control is shown in Figure 3.2 (C) and had GAL4

normal locomotor activity during both LD and DD cycles. Figure 3.2 (D-F) depicts the UAS-amon-RNAi controls, all of which displayed normal locomotor behavior under DD. Figure 3.2 (G-I) shows double plotted actograms for all experimental flies; those that have inherited both the clk-GAL4 and UAS-amon-RNAi elements. All experimental flies looked normal under DD.

III. Locomotor Behavior and Sleep Using *UAS-Dicer2* and *w*; UAS-amon-RNAi^{28b};+*

In an attempt to further knock down *amontillado* expression, GAL4 lines combined with UAS-Dicer2 were used. Dicer is an endonuclease and initiates the RNA-induced silencing complex (RISC) by cutting mRNA into siRNAs (Pratt and MacRae 2009). In this case, dicer2 will initiate RISC for *amontillado* mRNA degradation, thereby promoting knockdown. The two Dicer2 containing fly lines that I used were *UAS-Dicer2; +; Tim(UAS)-GAL4* and *UAS-Dicer2; +; cry(13)-GAL4/+.* The *tim(UAS)-GAL4* is expressed everywhere in the CCNN (Emery et al. 1998) and the cry-GAL4 is expressed in the DN1p, DN3, DN1, s-LNv, l-LNv, LNd, thus half of the CCNN (Emery et al. 2000). Virgin females of these lines were crossed to males from the line *w*; UAS-amon-RNAi^{28b};+* and put in an LD cycle at 25°C. The UAS-amon-RNAi control is depicted in Figure 4.1 (A) and showed normal anticipation during the LD cycle. The cry(13)-GAL4 and tim-GAL4 controls are illustrated in Figure 4.1 (B-C). These GAL4 controls also showed normal anticipation before lights on and lights off. Experimental flies are illustrated in Figure 4.1 (D-E), and demonstrated normal locomotor behavior.

The tabulated behavioral data for experimental flies and controls under constant darkness and temperature (DD) are shown in Table 2, where data from constant dark

conditions was analyzed. The average free running period length (tau), standard deviation, and % rhythmiciy was calculated. Genotypes *w*^{1118}*;UAS-amon-RNAi*^{28b}/+;+, *w*^{1118}*;+;tim(UAS)-GAL4/+, w*^{1118}*;+;cry(13)-GAL4/+,* and *UAS-Dicer2;UAS-amon-RNAi*^{28b}*/+;cry(13)-GAL4/+* showed to have a tau of near 24 hours and high % rhythmicities; similar to that of wild-type flies. The *per*^{01} mutant and experimental genotype *UAS-Dicer2;UAS-amon-RNAi*^{28b}*;tim(UAS)-GAL4* showed low rhythmicities (25.86%, and 36.84% respectively) and a tau of approximately 22.6. The low % rhythmicity is consistent with the behavior of *per*^{01} arrhythmic flies (Konopka and Benzer 1971). The low % rhythmicity of the experimental genotype *UAS-Dicer2;UAS-amon-RNAi*^{28b}*;tim(UAS)-GAL4* indicates arrhythmicity. This leads to the conclusion that Dicer2 overexpression and the expression of the *amontillado* RNAi construct had an effect on the locomotor behavior of the experimental flies under constant conditions, significantly reducing the proportion of rhythmic flies under DD.

To view a fly's locomotor behavior throughout the length of the experiment, the activity of single flies was visualized in double plotted actograms. Figure 4.2 (A) shows a double plotted actogram for the UAS-amon-RNAi control with normal locomotor behavior, even after the switch to contants conditions. Figure 4.2 (B-C) illustrates double plotted actograms for the GAL4 controls, which have normal locomotor activity. The experimental flies (Fig. 4.2, D-E) showed normal behavior during the LD cycle, but were arrhythmic during the length of constant darkness. These experimental flies are single fly examples of the arrhythmic portion of the sample.

Sleep graphs were generated for the experimental crosses, and were compared to the positive control *w*^{1118}*.* Figure 4.3 (A) compares *w*^{1118} and the *per* mutant, *yw,*

per^{01}. *w*^{1118} maintains its sleep rhythm well after being switched to constant darkness, whereas *yw, per*^{01} immediately loses its sleep rhythm. *w*^{1118}*; UAS-amon-RNAi*^{28b}*;*+ (Fig. 4.3, B) and *w*^{1118}*;+;cry(13)-GAL4/*+ (Fig. 4.3, C) controls displayed a clear sleep rhythm under DD conditions. Figure 4.3 (D) compares *w*^{1118} and *UAS-Dicer2; UAS-amon-RNAi*^{28b}*; cry(13)-GAL4* flies, which both display clear sleep rhythms under constant darkness. Figure 4.4 (A) compares *w*^{1118} and the negative control *yw, per*^{01} again for comparison purposes. Figure 4.4 (B) shows the sleep rhythm for *w*^{1118} and *w*^{1118}*; UAS-amon-RNAi*^{28b}*/+; +,* and the sleep rhythms persist in constant darkness.. Figure 4.4 (C) compares *w*^{1118} and *w*^{1118}*;+;Tim(UAS)-GAL4/+;* the control is able to keep a sleep rhythm. Figure 4.4 (D) compares *w*^{1118} and experimental flies *UAS-Dicer2; UAS-amon-RNAi*^{28b}*; Tim(UAS)-GAL4.* The experimental flies start out in sync with *w*^{1118}, but start to plateau at the end of the DD cycle. It is possible that this unusual plateau at the end of the DD cycle could be due to the RNAi knock down of *amontillado,* and has affected its sleep rhythm.

Discussion

Drosophila melanogaster is a great model system to further understand circadian rhythms and the roles that the CCNN neurons play in the control of rhythmic behaviors and physiology. These clock neurons release different neuropeptides, which are responsible for the fruit fly's circadian rhythms. Currently, there has only been a few neuropeptides (PDF, NPF, ITP, sNPF, IPNamide, DH31) that have been identified in the *D. melanogaster* CCNN (Renn et al. 1999; Shafer et al. 2006; Johard et al. 2009), and there are certainly many more yet to be discovered. The question I was trying to answer throughout this project is "What are the effects of preventing specific groups of

clock neurons from releasing neuropeptides on the circadian behavioral rhythms of the fly?"

I observed the effects on *D. melanogaster* behavior (locomotor activity and sleep) caused by the RNAi knockdown of *amontillado* in CCNN neurons. This was done by attempting to knock down the expression of an enzyme required for neuropeptide processing, *amontillado*, instead of knocking individual neuropeptides in the different classes of the CCNN. This allowed me to prevent the release of bioactive neuropeptides in certain regions of the CCNN, rather than inhibiting only the neuropeptides currently known to be expressed in clock neurons. To knock down the gene expression of *amontillado* within specific classes of the CCNN neurons, I used the GAL4/UAS-RNAi system.

A lethality assay was used to determine which *amontillado* RNAi lines would be effective at knocking down *amontillado* expression in the CCNN. It was concluded from this experiment that three of the four *amontillado* RNAi lines used, were effective (Table 1). One of the three accepted *amontillado* RNAi lines that was mainly used for future experiments was *w*; UAS-amon-RNAi^{28b};+.* This *amontillado* RNAi line has shown to knock down adipokinetic hormone (AKH) in *D. melanogaster* larvae (Rhea et al. 2010). AKH is responsible for increasing sugar levels in *D. melanogaster*, and according to Rhea et al. (2010), sugar levels were low in larvae with *amontillado* knock down. Because of the success of this experiment, *w*; UAS-amon-RNAi^{28b};+* was used.

The endeavor of this project was to knock down *amontillado* gene expression in the *Drosophila* clock and determine how behavioral rhythms like locomotor activity and sleep are affected. Early experiments using *clk-GAL4* and different *UAS-amon-RNAi*

lines showed normal locomotor behavior during an LD cycle. These negative results could have been a result of the various UAS-amon-RNAi lines not being strong enough to knock down *amontillado* expression in the clock neurons. Another possibility is that the clk-GAL4 driver did not target all clock neurons. Future work could address this by using immunocytochemistry and use anti-sera raised against *amontillado* and circadian neuropeptides, like PDF, to determine if colocalization is present or not.

To increase the knockdown of *amontillado* expression, the *amontillado* RNAi line was combined with *UAS-Dicer2 GAL4* elements. *Dicer2* is involved in the first step of the RNA-induced silencing complex (RISC) (Pratt and MacRae 2009). The added effects of using *UAS-Dicer2* seemed to have an effect in *Drosophila* free-running behavior during constant conditions (Figure 4.2 D-E; Table 2) and sleep behavior (Figure 4.4 D). It is possible that preventing *amontillado* expression affected these rhythms.

I was not able to determine if knock down of *amontillado* expression really occurred. Future work could address this by using immunocytochemistry and using anti-sera raised against *amontillado*. It is possible that in my experiments *amontillado* expression was not successfully knocked down, if it were, pdf^{01} phenotypes would at least be observed in experiments involving cry-GAL4. The pdf^{01} mutant has an inability to anticipate lights on during LD and shows both an arrhythmic and fast-running period during DD (Renn et al. 1999). The only experimental flies where this was observed were the *UAS-Dicer; UAS-amon-RNAi*^{28b}*; tim(UAS)-GAL4* (Table 2). Precursors of neuropeptides go through various modifications during peptide processing by the enzymes carboxypeptidase D (SILVER) and amidating enzymes (PHM and PAL)

(Wegener et al. 2011). Understanding more about the other enzymes involved in neuropeptide processing can lead to better prevention of neuropeptide release onto the circadian rhythms of the fly.

Figures and Tables

TABLE 1: Lethality Assay Observations				
Cross	**Curly Wing Phenotype (N)**	**Straight Wing Phenotype (N)**	**X^2**	**Result**
y1w*; P{ACT5C-GAL4}F01/CyO, y+; + X w*; +; P{UAS-amon-RNAi} 78b	118	2	112.14	Reject
y1w*; P{ACT5C-GAL4}F01/CyO, y+; + X Y1v1; +; P{TRiP.HM05071}attP2	89	105	1.32	Accept
y1w*; P{ACT5C-GAL4}F01/CyO, y+; + X W*; P{UAS-amon-RNAI} 69h: +	92	1	89.04	Reject
y1w*; P{ACT5C-GAL4}F01/CyO, y+; + X W*; UAS-amon-RNAi^{28b}; +	112	1	109.04	Reject

Table 1: Lethality Assay Observations. Actin-GAL4 virgin females were crossed to males of four different UAS-amon-RNAi lines. Flies of each phenotype, curly wings or straight wings, were counted for each cross. The chi square value for each genotype was calculated using the Chi Square formula in order to accept or reject the null hypothesis. Null Hypothesis: There will be equal numbers of straight and curly-winged flies.

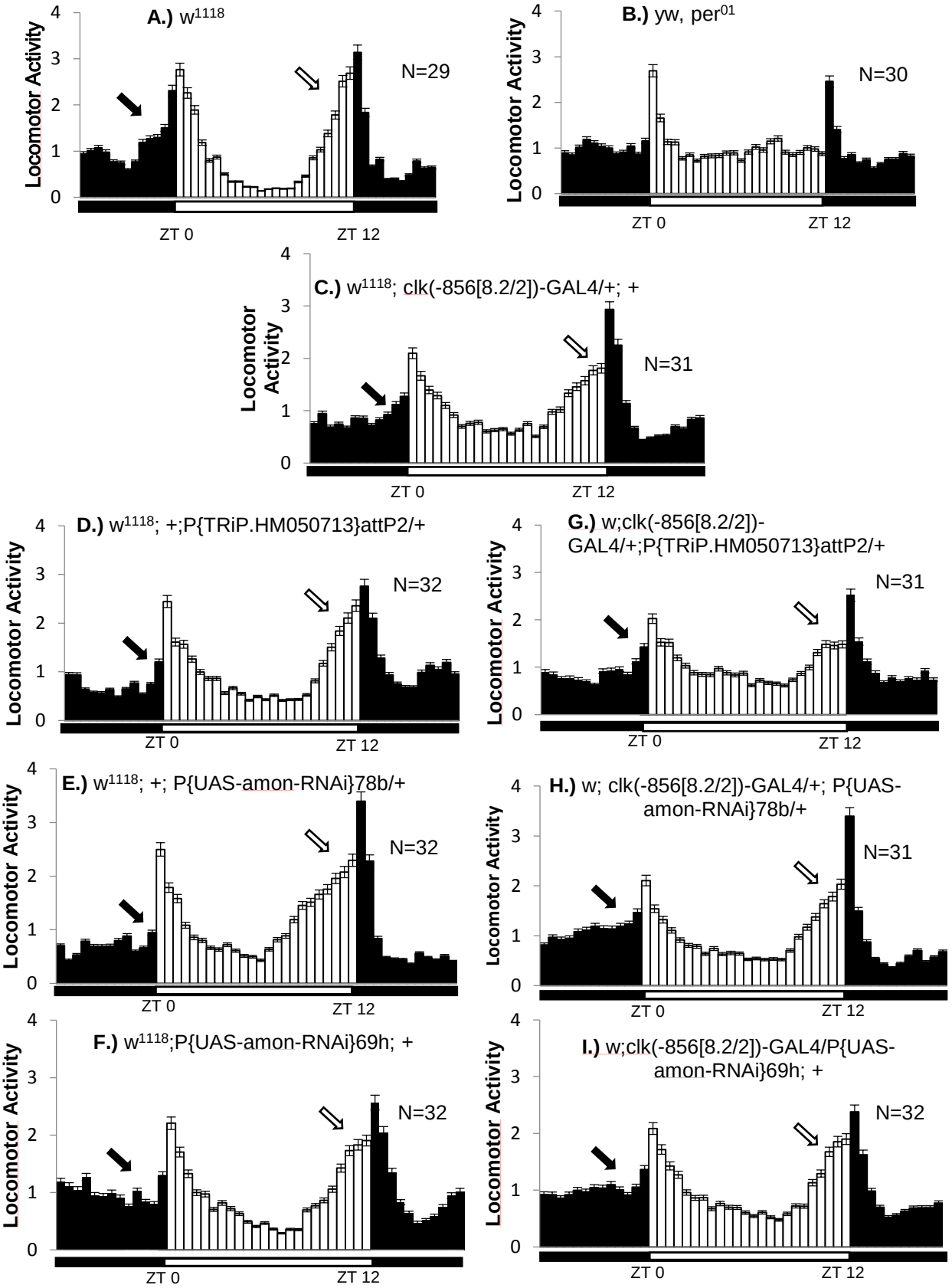
A.) w1118
N=29
B.) yw, per01
N=30
C.) w1118; clk(-856[8.2/2])-GAL4/+; +
N=31
D.) w1118; +;P{TRiP.HM050713}attP2/+
N=32
G.) w;clk(-856[8.2/2])-GAL4/+;P{TRiP.HM050713}attP2/+
N=31
E.) w1118; +; P{UAS-amon-RNAi}78b/+
N=32
H.) w; clk(-856[8.2/2])-GAL4/+; P{UAS-amon-RNAi}78b/+
N=31
F.) w1118;P{UAS-amon-RNAi}69h; +
N=32
I.) w;clk(-856[8.2/2])-GAL4/P{UAS-amon-RNAi}69h; +
N=32
Locomotor Activity
ZT 0
ZT 12

Figure 3.1: Eduction Plots for Locomotor Activity: Testing Various UAS-amon-RNAi Lines. Flies were put in a 12:12hr LD cycle for 5 days and switched to constant darkness (DD) cycle for 8 days at a constant temperature of 25°C. **A.)** *w^{1118}*: eduction plot of an individual wild-type fly. **B.)** *yw, per^{01}*: eduction plot for *per^{01}* mutant flies. Note the absence of anticipatory peaks of activity before lights-on and lights-of **C.)** An eduction plot for the clk-GAL4 control *w; clk(-856[8.2/2])-GAL4/+*: **D-F.)** Eductions plots for UAS-amon-RNAi elements *w^{1118};+;P{TRiP.HM050713}attp2/+, w^{1118};P{UAS-amon-RNAi}69h:+/+, and w^{1118};+;P{UAS-amon-RNAi}78b/+:*. **G-I)** Eduction plots for the experimental *w; clk(-856[8.2/2])-GAL4;P{TRiP.HM050713}attP2/+, w;clk(-856[8.2/2])-GAL4/P{UAS-amon-RNAi}69h;+/+,* and *w;clk(-856[8.2/2])-GAL4/+;P{UAS-amon-RNAi}78b/+* flies. Black bars represent darkness, while white bars represent light. **ZT**-zeitgebers time, **ZT 0**–lights-on. **ZT 12** – lights-off. N indicates the number of flies observed.

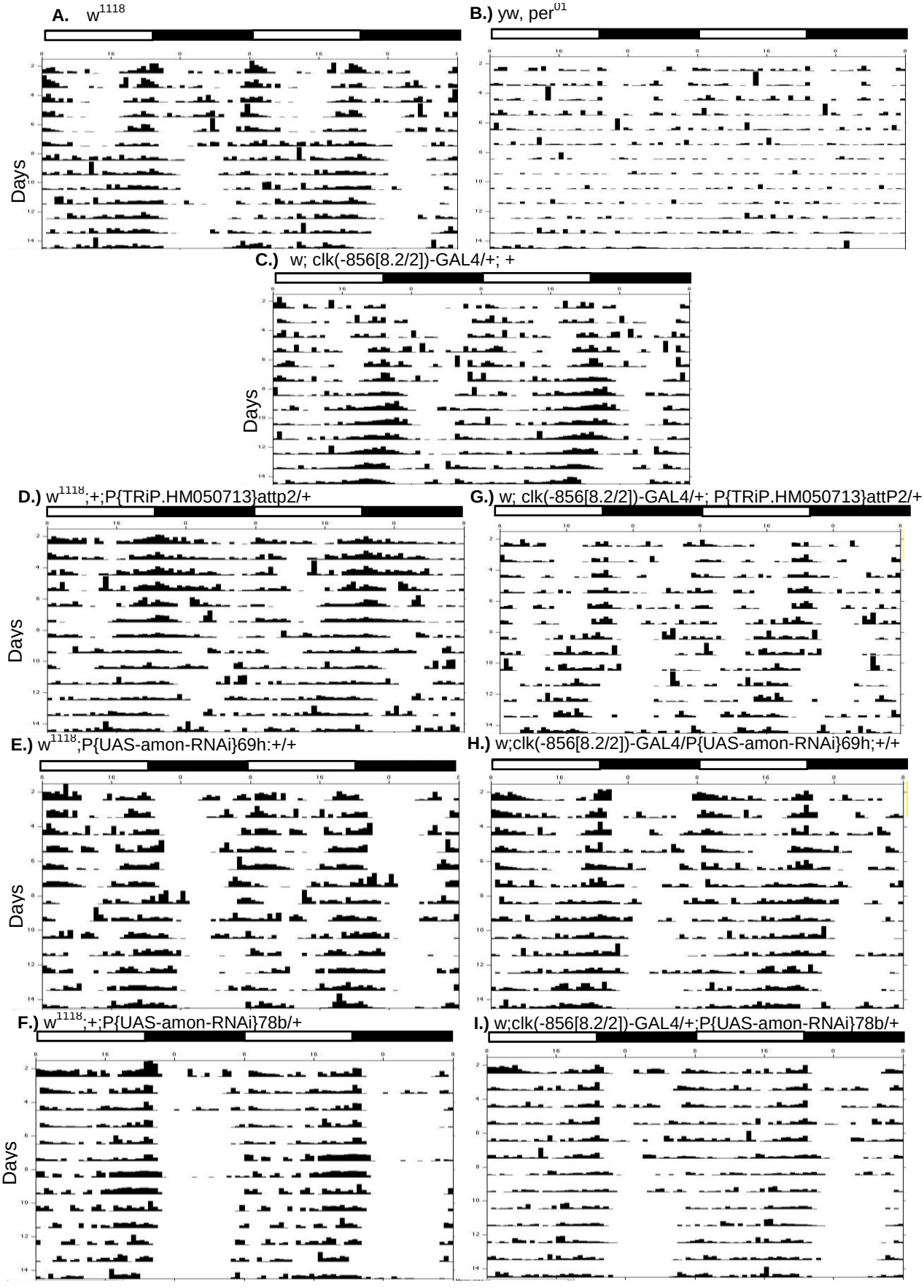
A. w1118
B.) yw, per01
C.) w; clk(-856[8.2/2])-GAL4/+; +
D.) w1118;+;P{TRiP.HM050713}attp2/+
G.) w; clk(-856[8.2/2])-GAL4/+; P{TRiP.HM050713}attP2/+
E.) w1118;P{UAS-amon-RNAi}69h:+/+
H.) w;clk(-856[8.2/2])-GAL4/P{UAS-amon-RNAi}69h;+/+
F.) w1118;+;P{UAS-amon-RNAi}78b/+
I.) w;clk(-856[8.2/2])-GAL4/+;P{UAS-amon-RNAi}78b/+
Days

Figure 3.2: Double Plotted Actograms of Flies with Different UAS-amon-RNAi Elements. Flies were put in a 12:12 hr LD cycle for 5 days and switched to DD for 8 days. **A.)** *w^{1118}*: double plotted actogram of an individual wild-type fly. **B.)** *yw, per^{01}*: double plotted actogram of an individual per^{01} mutant. Note the lack of an obvious rhythm in locomotor activity. **C.)** Double plotted actogram for the *w; clk(-856[8.2/2])-GAL4/+* control. **D-F.)** Double plotted actograms for the UAS-RNAi elements *w^{1118};+;P{TRiP.HM050713}attp2/+, w^{1118};P{UAS-amon-RNAi}69h:+/+, and w^{1118};+;P{UAS-amon-RNAi}78b/+*. **G-I)** Double plotted actograms for the *w; clk(-856[8.2/2])-GAL4;P{TRiP.HM050713}attP2/+, w;clk(-856[8.2/2])-GAL4/P{UAS-amon-RNAi}69h;+/+,* and *w;clk(-856[8.2/2])-GAL4/+;P{UAS-amon-RNAi}78b/+* experimental flies. White bars indicate day during LD cycle, black bars indicate night. DD conditions started on day six of the experiment. N indicates the number of flies observed.

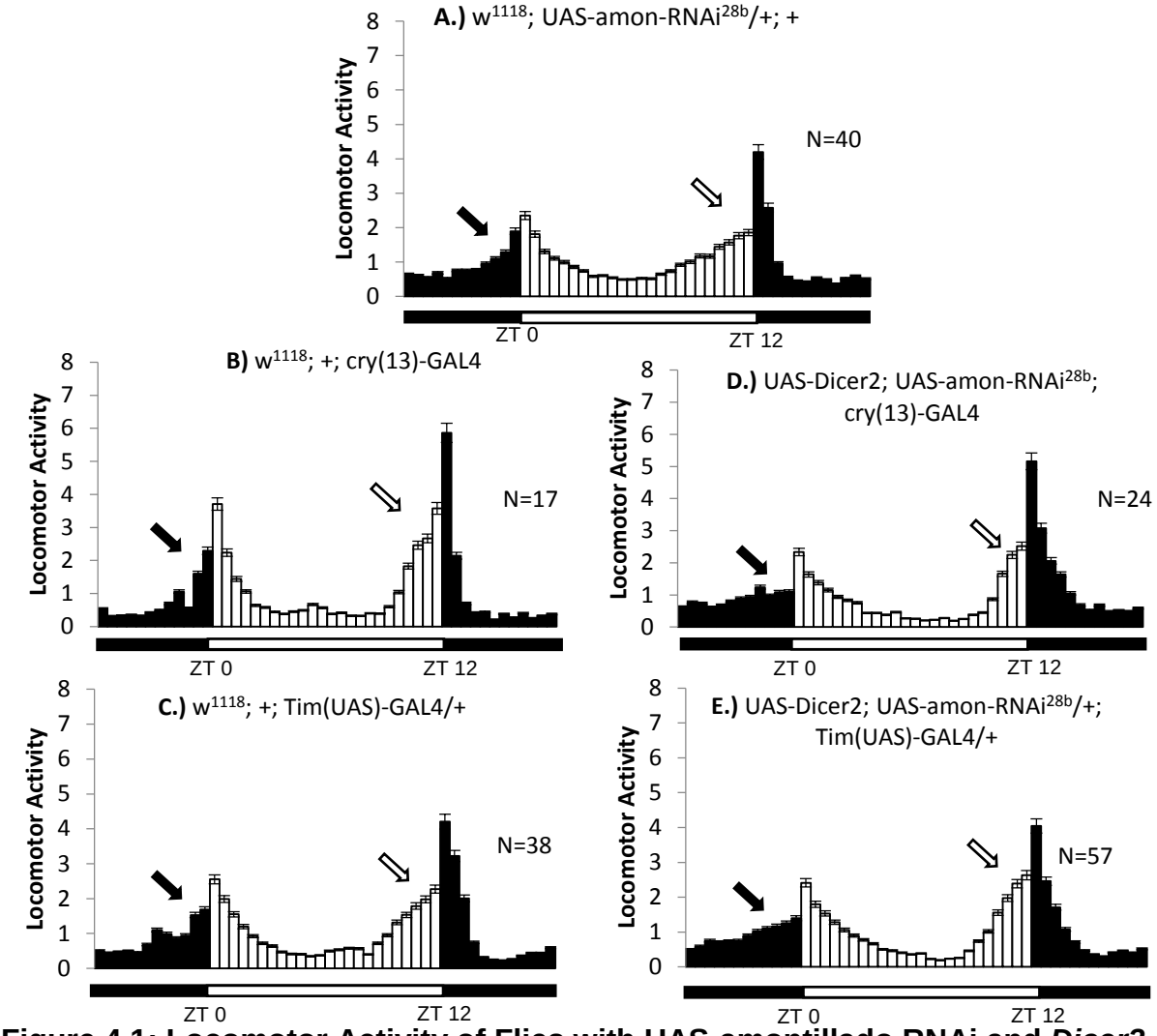

Figure 4.1: Locomotor Activity of Flies with UAS-amontillado RNAi and *Dicer2* Expression in the CCNN. Arrows indicate morning (black) and evening (white) anticipation. A**.)** Eduction plot for the UAS-amon-RNAi line control *w^{1118}; uas-amon-RNAi^{28b}; +*: **B-C.)** Eduction plots for the GAL4 controls *w^{1118}; +; Tim(UAS)-GAL4/+* and *w^{1118}; +; cry(13)-GAL4/+*: **D-E.)** Eduction plots for the *UAS-Dicer2; UAS-amon-RNAi^{28b}/+; cry(13)-GAL4* and *UAS-Dicer2; UAS-amon-RNAi^{28b}/+; Tim(UAS)-GAL4/+*: experimental flies. Data were pooled from two independent experiments. Black bars are represent darkness, while white bars represent light. **ZT**- zeitgebers time, **ZT 0** – lights-on. **ZT 12** – lights-off. N indicates the number of flies observed.

Table 2: Average Period and % Rhythmicity of UAS-Dicer2-GAL4 Lines					
#	Genotype	N	Average Tau (hr)	SD	% Rhythmicity
1	w^{1118}	54	23.86	0.5506	81.48%
2	yw, per^{01}	58	22.64	6.6962	25.86%
3	w^{1118}; UAS-amon-RNAi^{28b}/+; +	40	23.97	0.4649	92.50%
4	w^{1118}; +; Tim(UAS)-GAL4/+	38	24.18	0.4152	97.37%
5	w^{1118}; +; Cry (13)-GAL4/+	17	24.50	0.3809	100.00%
6	UAS-Dicer2; UAS-amon-RNAi^{28b}/+; Tim-GAL4/+	57	22.69	1.8567	36.84%
7	UAS-Dicer2; UAS-amon-RNAi^{28b}/+; Cry (13)-GAL4/+	24	23.86	0.544705	62.50%

Table 2: Free running rhythms in flies co-expressing Amontillado RNAi and Dicer2 in the CCNN. Flies were left in constant dark conditions for 8 days at 25°C, only the first 7 days were analyzed. The average tau (period length), standard deviation, and % rhythmicity was calculated using Chi-square periodogram analysis. Data was pooled from two independent experiments.

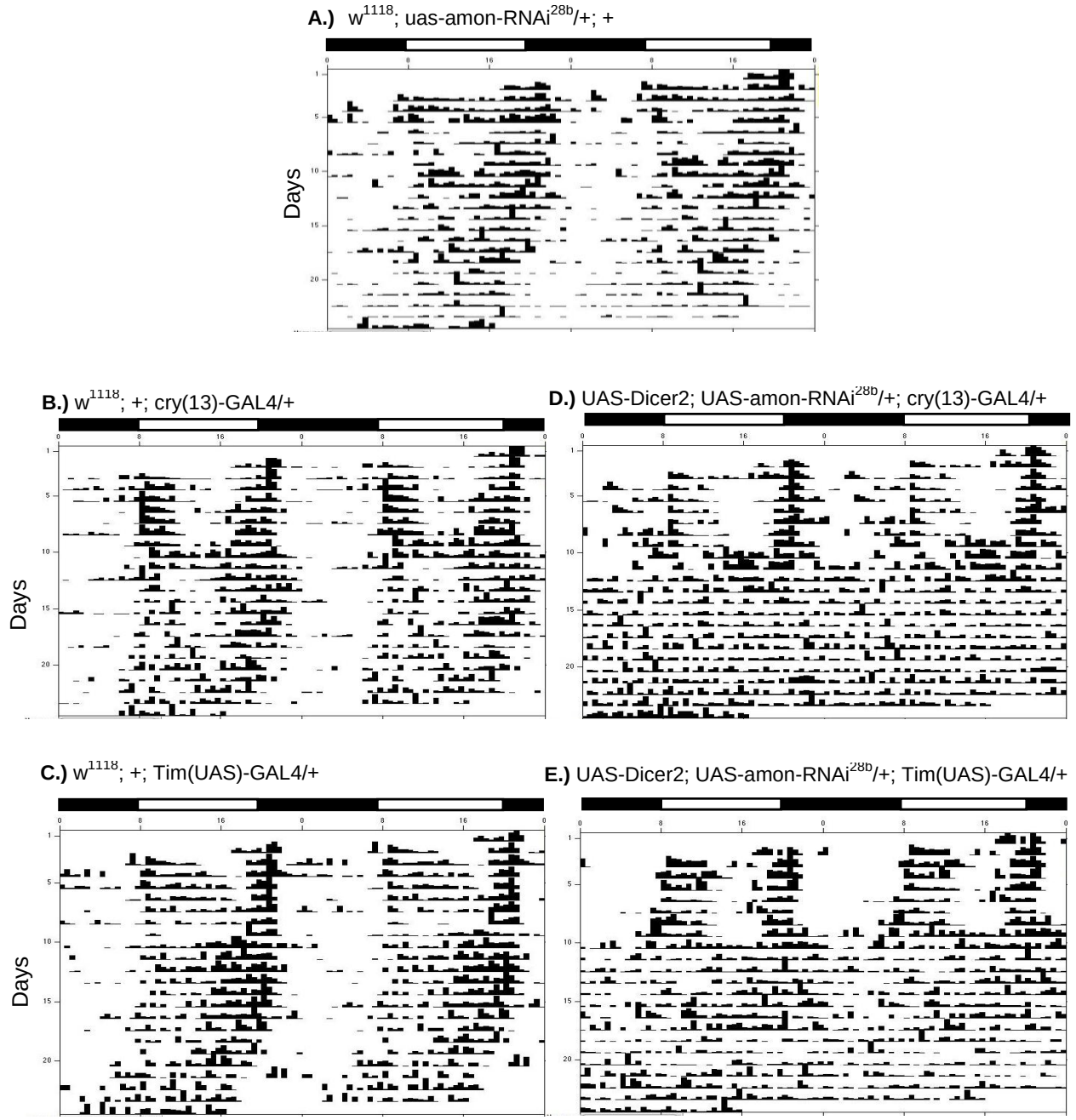

Figure 4.2: Free Running Behavior of Individual Flies with an RNAi Knock Down of *amontillado* and Enhanced *Dicer2* Expression. Flies were put in a 12:12 hr LD cycle for 5 days and switched to a 12:12 hr LD cycle for 8 days at a constant temperature of 25°C. A**.)** *w^{1118}; uas-amon-RNAi^{28b};* +: Double plotted actogram for the UAS-amon-RNAi line control. **B-C.)** *w^{1118}; +; Tim(UAS)-GAL4/*+ and *w^{1118}; +; cry(13)-GAL4/*+: Double plotted actograms to control for the GAL4 lines used. **D-E.)** *UAS-Dicer2; UAS-amon-RNAi^{28b}/+; cry(13)-GAL4* and *UAS-Dicer2; UAS-amon-RNAi^{28b}/+; Tim(UAS)-GAL4/*+: Double plotted actograms for the experimental flies White bars indicate day during LD cycle, black bars indicate night. DD conditions started on day six of the experiment. N indicates the number of flies observed.

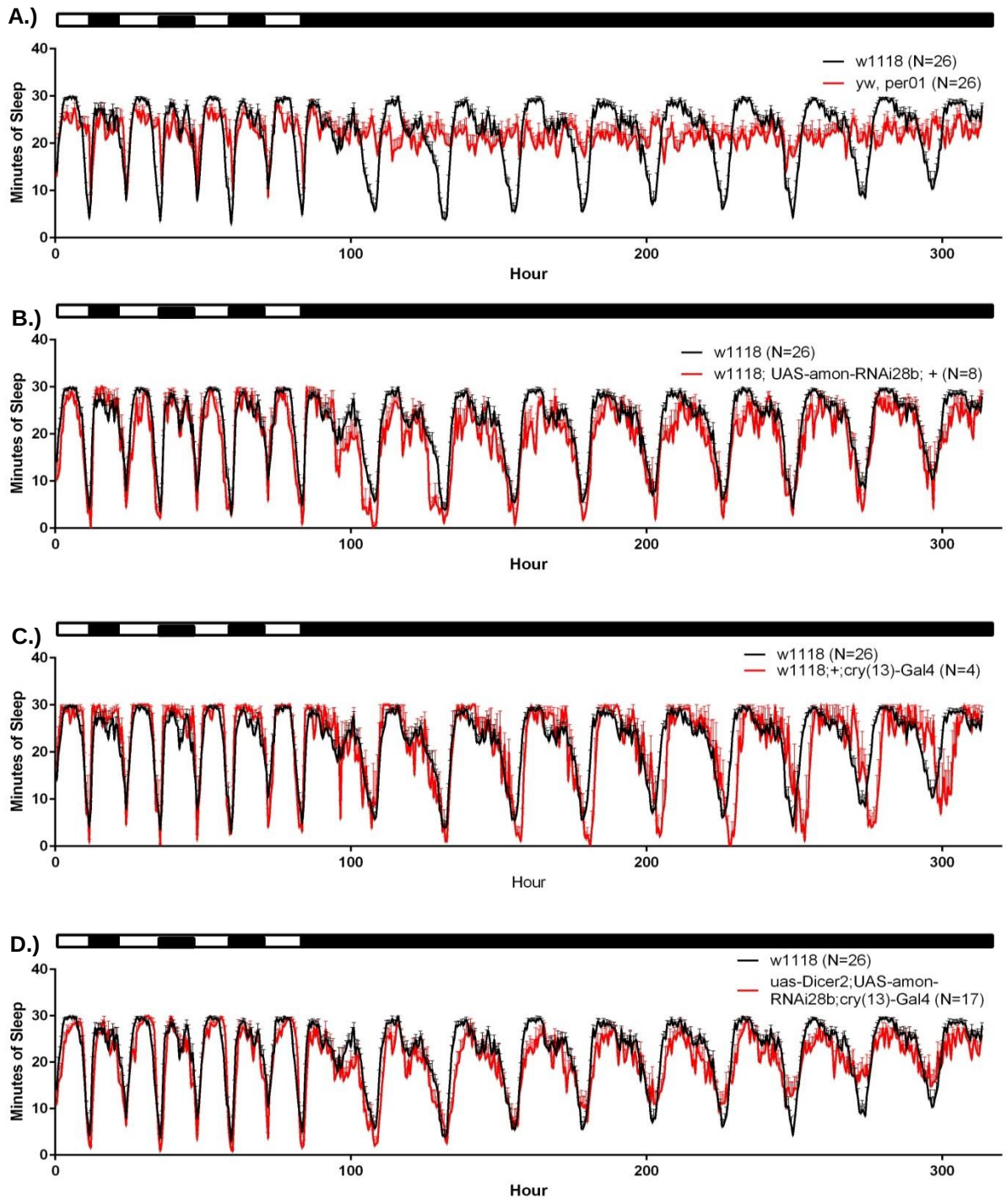

Figure 4.3: Sleep Behavior of *amon* RNAi Knock Down in *cry-GAL4* Expressing Cells. Sleep graphs of experimental flies and controls displaying average sleep duration per hour for 4 days of LD and 9 days of DD. The genotypes of each plot are indicated above. Sleep is plotted as minutes of sleep per hour. White bars represent the light and black bars represent darkness.

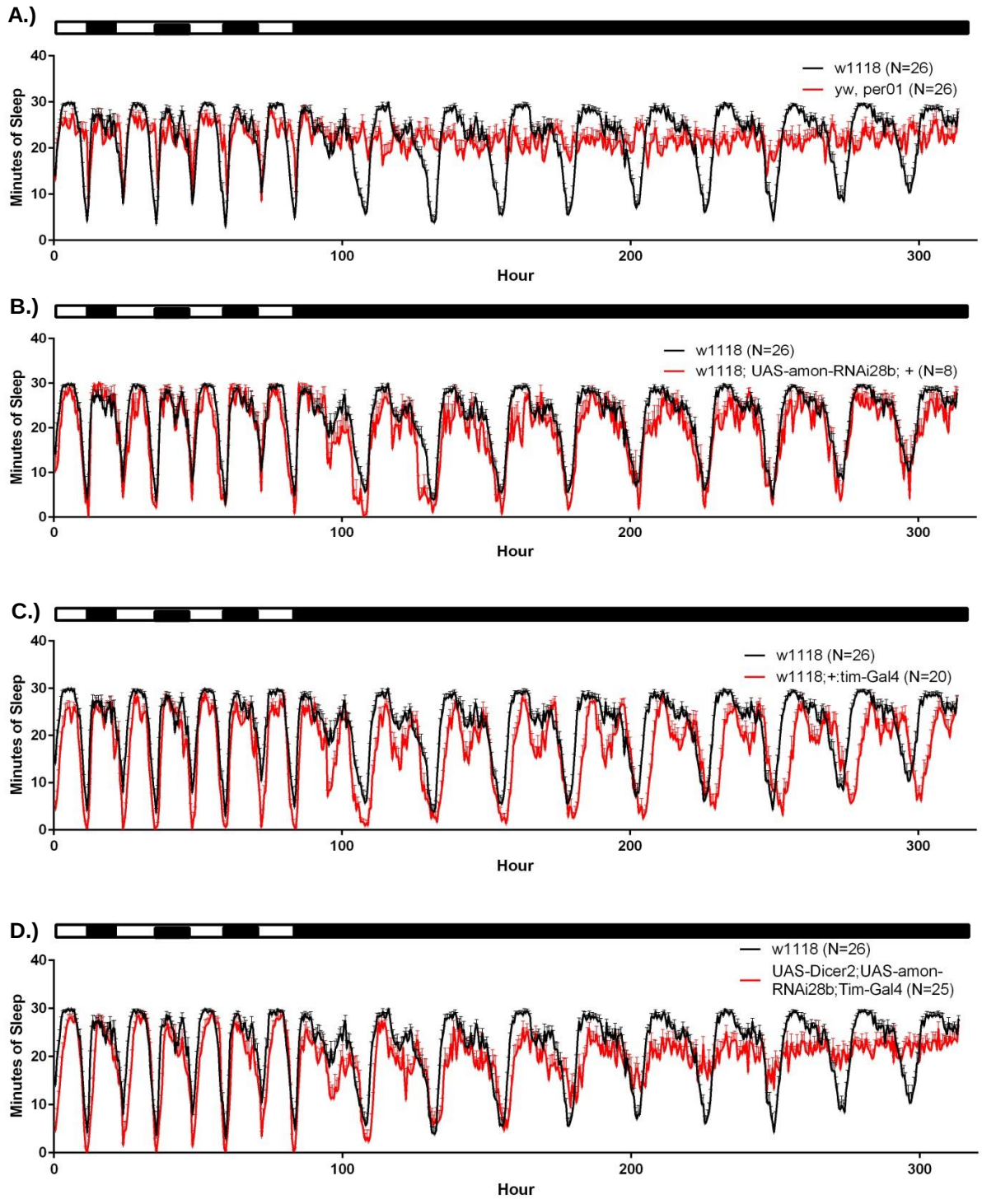

Figure 4.4: Sleep Behavior of *amon* RNAi Knock Down in *tim(UAS)-GAL4* Expressing Cells. Sleep graphs of experimental flies and controls displaying average sleep duration per hour for 4 days of LD and 9 days of DD. The genotypes of each plot are indicated above. Sleep is plotted as minutes of sleep per hour. White bars represent the light and black bars represent darkness.

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