UCL CoMPLEX Case Presentation 2:

Experimental and computational analysis of the Drosophila circadian clock

Author: Matthew Topping

Supervisors: Dr. Joerg Albert
Prof. Peter Dayan

Abstract

Variability between the activity levels of individual animals in circadian clock experiments is greatly pronounced, and the data that is collected can often include large amounts of noise. In order to investigate this noise and the effects it can have on models of activity, an experiment involving four different varieties of *Drosophila melanogaster* was conducted. This allowed for the exploration of the activity patterns of *tilB* mutants, whose ablation of chordotonal organ function makes them an interesting area of research. Behavioural patterns of flies between entrainment and free run periods were investigated and where found were used as evidence in an attempt to build a model of fly activity for each of the fly types. Finally future avenues for potential experiments were noted, especially with regards to gaining more detailed information on fly activity habits.

Contents

List of figures

1. The circadian clock

The presence of a circadian, or 'biological', clock within an organism grants a large advantage to that being [Ouyang *et al.*, 1998] because time dependent functions within the entity can continue without the need for external stimuli. The benefits of the ability to anticipate, and thus undergo preparations for, significant environmental events such as the rising of the sun are clear [Piggins and Guilding, 2011], not least the removal of an otherwise complete dependence on occasionally unreliable stimuli to react to foreseeable temporal changes.

The circadian system in mammals is usually dominated by the suprachiasmatic nuclei, located in the hypothalamus, though additional oscillators do exist in other parts of the body [Hughes and Piggins, 2012]. Fortunately the basic genetic mechanisms are similar in mammals and insects [Glossop, 2011], which allows for in depth studies to be conducted on species such as *Drosophila melanogaster* [Risau-Gusman and Gleiser, 2012]. Having a clock period of approximately 24 hours is considered important for good health, with malfunctioning clocks being linked to diseases such as narcolepsy [Milhiet *et al.*, 2011] as well as shortened lifespans in mice [Libert *et al*., 2012].

Although entrainment of the clock by *zeitgeber* ('time givers') can be achieved in a number of different ways, such as specific eating times [Hart, 2013] or vibrational stimuli [Simoni *et al.*, submitted], light entrainment is probably the biggest area of research in this field. *Drosophila* can be entrained after just a few days of light/dark cycles into a specific biological rhythm, though some drift does occur eventually after the animals enter free run (during which the insect is kept in complete darkness).

Drosophila can be entrained by vibrational cycles because they have small ciliated stretch receptors known as chordotonal organs, which are involved not only in proprioception (which, for example, is involved in monitoring the relative position of each limb) but also exteroception (which includes checking vibrations resulting from wind or noise). They have also been strongly implicated in circadian clock entrainment by temperature, as mutants with no chordotonal organs were not able to be entrained to a temperature cycle [Sehadova *et al.*, 2009]. In the same experiment, mutant flies with chordotonal organs but lacking a circadian clock did not become entrained by the stimulus regime, although they did respond to it.

There is considerable variability observed between the activity levels and daily cycle lengths of individual flies, and some flies seem to have more noise than others during entrainment and free run. With this in mind, an experiment investigating more closely the impact of long term photic entrainment on noise and activity levels in various fly types would be useful with respect to improving the quality of mathematical modelling for fly behaviour. By including fly strains lacking chordotonal organs it would also be possible to look at the effect of a lack or proprioception on activity levels when compared to regular flies.

2. Experimental aims

In order to fully explore the possible changes in activity, amongst other things, that are the result of a lack of chordotonal organs it was decided to include two separate varieties of fly that have reduced or no chordotonal function for different reasons. The types selected were touch insensitive larvae B (*tilB)* mutants, which show a complete ablation of function in their chordotonal organs [Kavlie *et al.*, 2010], and Canton S (wild-type) flies that had been fed the insecticide pymetrozine, which is able to affect all chordotonal organs in the fly provided that the concentration is sufficient [Ausborn, *et al.*, 2005]. The method of action of pymetrozine is unknown, but its abilities have been tested beyond reasonable doubt [Sehser, 2002].

Canton S flies were necessary for inclusion in the experiment, not only to investigate noise and variability in activity but also to act as a control group for the purposes of comparison. To act as a further control, *tilB* flies that have been fed pymetrozine were also included, even though the

insecticide should have a reduced effect on the animal's proprioceptive and exteroceptive abilities.

The inclusion of flies lacking proprioception is important not just because of the removal of a possible entrainment pathway with the removal of chordotonal organs. The circadian system is multi-directional, and does not simply follow a simple chain of a stimulus entraining a biological clock, which results in an output that controls biological rhythms: clearly the output, such as movement, could itself have an impact on the circadian clock. Therefore, since *tilB* flies are less able to sense their own body movements than wild type flies, it is possible to study the potential impact of this lack of proprioception in terms of the flies activity and diurnal rhythms.

Usually the entrainment period for flies is around three or four days. By increasing this time span to ten days it was hoped that problems such as noise and masking (whereby stimuli directly affect the animal without having any impact on the circadian clock) can be looked at in closer detail because of the increased amount of data available. Photic entrainment was used not only for simplicity but also because *tilB* flies cannot be entrained via vibrational stimuli [Simoni *et al.*, submitted].

3. Materials and methods

Chemicals and their application: Pymetrozine with approximately 99.5% purity was sourced directly from Syngenta, Basel, Switzerland.

The pymetrozine doped feeding assays were prepared according to a recipe obtained from a Syngenta employee (see appendix I). Briefly, pymetrozine was dissolved in a solvent mixture and then added to the surface of food wells (which contained a mixture of sugar, agar and water). These were then allowed to dry for over an hour before being ready for use.

Flies: The experiment was carried out on four separate varieties of male *Drosophila melanogaster* flies: Canton S, Canton S that had been exposed to pymetrozine (following the procedure outlined in appendix I), *tilB* mutants and *tilB* that had been exposed to pymetrozine. All flies selected for inclusion in the experiment had undergone eclosion between four and seven days before the experiment began. All flies were provided by Ryan Kavlie, working at the Albert laboratory at the UCL Ear Institute.

Experimental preparation: 60 flies (30 Canton S, 30 *tilB* mutants) were placed in groups of ten according to genotype into the food assays. The flies were left to feed for one day before being removed, and were then placed on non-doped food for two days before the experiment began. Upon removal of the flies from the assays, it was noted that one fly of each genotype had died.

Experimental set-up: 96 flies (made up of 24 flies for each of the four subgroups) were placed in tubes which contained sufficient food for the experiments duration on one side – this food was standard agar food that did not contain pymetrozine. These tubes were then loaded into three separate Trikinetics Drosophila Activity Monitor (DAM) 2 activity monitors, which are able to monitor 32 flies at once. The monitors shine an infra-red beam down the centre of each tube and count the number of times the beam is broken by the fly in order to calculate how many times each fly move along the tube. The monitors sum the count data into bins of an appropriate time length and send the data to a computer for analysis (see Data analysis section below).

Mechanical response measurements: In order to verify that the insecticide had had the desired effect on the flies, measurements of the antennal sound receiver's vibrations in response to sound were taken. These recordings were taken following the procedures described in the paper by Albert *et al.* [Albert *et al*., 2006] and with the help of Nerissa Marziano. A brief description of their importance and results can be found in Appendix II.

Entrainment details: All flies followed a ten day entrainment period of 12 hours of light

(commencing at 05:00 hours) and 12 hours of darkness. After this time period, the flies were monitored under constant darkness for another four days before the experiment was stopped. The experiment was carried out at room temperature and the activity monitors were placed in insulated boxes in order to minimise the effect of any unwanted light or temperature disturbances.

4. Data analysis

The data collected contains the number of beam breaks by each fly in bins of one minute over the entire experiment and the information is stored for each monitor, along with the date and time of the collection. These one minute intervals are then summed into bins of fifteen minutes. Unfortunately there are approximately 8 hours of missing data from the third day of the experiment, between 03:20 and 11:23 hours, because of problems with the computer that had been set up to collect the data. This was rectified as soon as possible and the data set is otherwise complete.

Of the 96 flies that were alive when the experiment started, 75 survived until the end of experiment – 19 Canton S, 22 Canton S that had been fed pymetrozine and 17 of each of the *tilB* groups. Flies were treated as dead if they had been completely inactive for at least 48 hours by the end of the experiment and this was verified after the experiment by checking individual flies. Deceased flies were completely excluded from all analyses.

There are a number of tools available in the FlyToolBox for Matlab so that the data can be analysed. Among the most useful are the ability to produce average actograms across a range of flies to illustrate average activity over a set period of time, and being able to create a range of histograms for different fly groups. The actograms shown below in figures 1 a) through 1 d) display activity in 15 minute intervals over the two weeks that the experiment ran for. Several actograms of random flies are presented in appendix III, whilst histograms for the mean activity across all flies of a specific type throughout entrainment or free run are displayed in appendix IV along with bar charts comparing the mean daily activity in entrainment with free run, and mean daily activity during entrainment in light and dark conditions. These actograms and histograms also have 15 minute bin sizes and include all data possible.

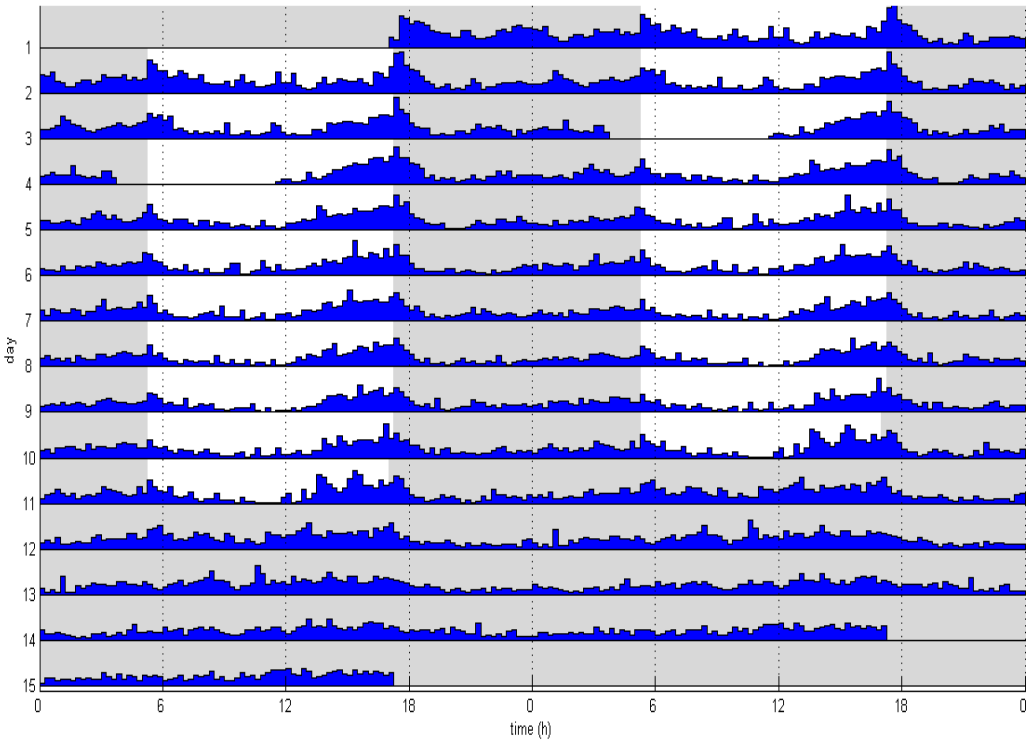


Figure 1 a) Average double plot actogram, including all days of available data, for all Canton S (wild type) flies that remained alive throughout the entire experiment

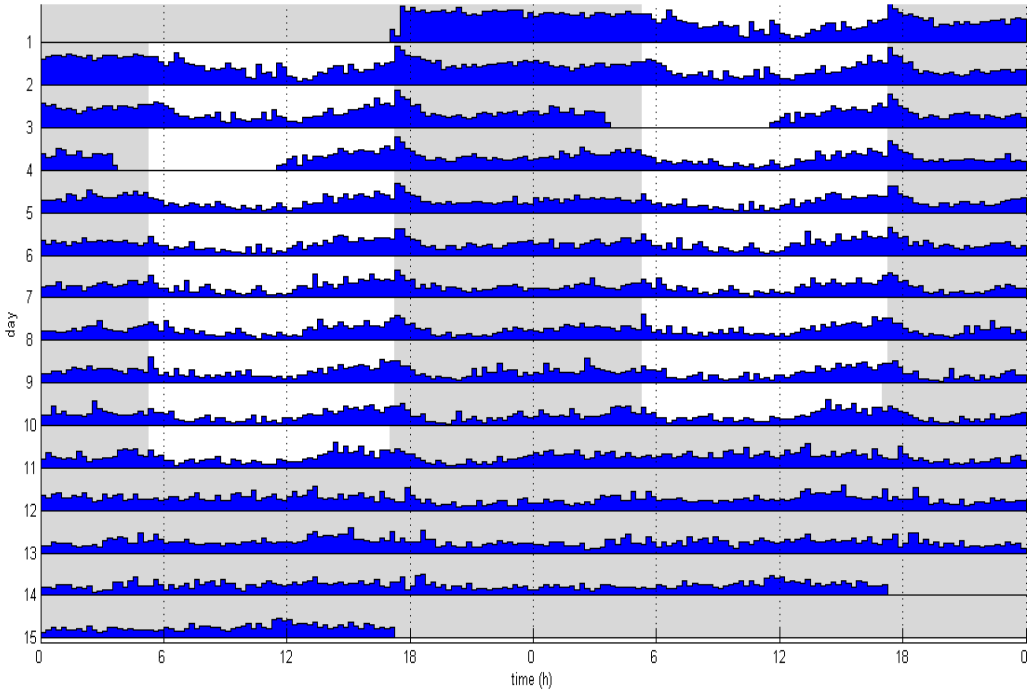


Figure 1 b) Average double plot actogram, including all days of available data, for all Canton S flies exposed to pymetrozine that remained alive throughout the entire experiment

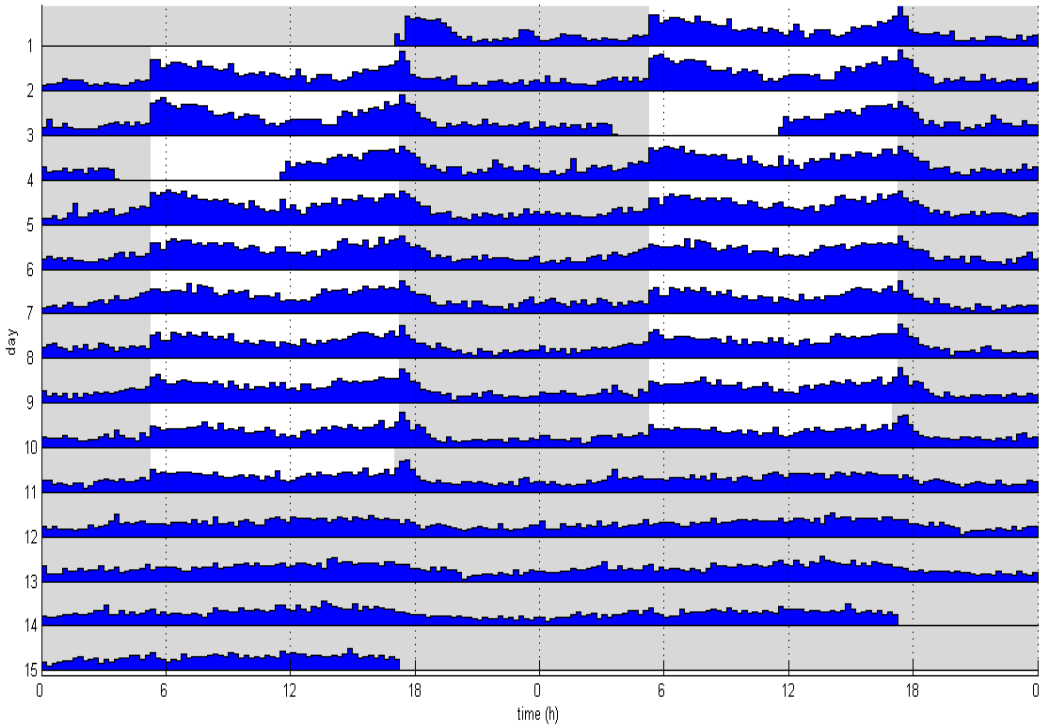


Figure 1 c) Average double plot actogram, including all days of available data, for all tilB flies that remained alive throughout the entire experiment

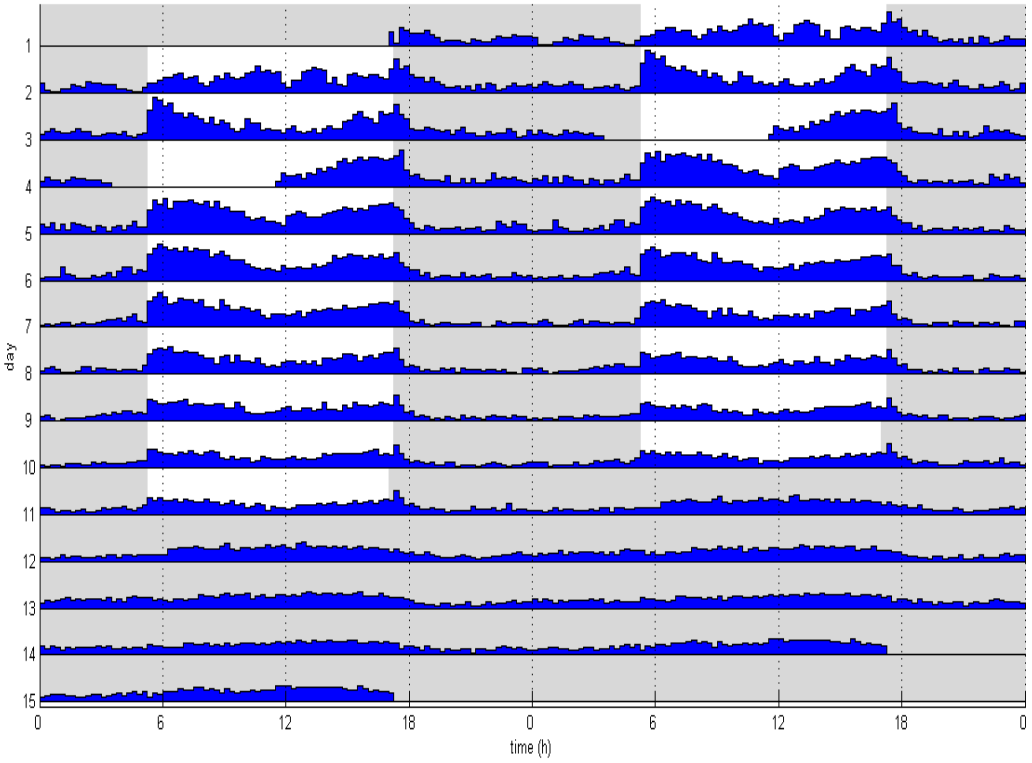


Figure 1 d) Average double plot actogram, including all days of available data, for all tilB flies exposed to pymetrozine that remained alive throughout the entire experiment

From the above figures, and those in appendix III, it is notable that average activity tends to decrease as the experiment progressed (almost certainly because of the increased ages of the flies). In addition to this both types of *tilB* mutants tended to have much greater average daily activities than Canton S flies during the light portion of photic entrainment, though activity level at 'night' is lower and possibly more in line with what might be expected from a wild-type fly. The variability in activity level between flies is very large, even when comparing flies of the same genotype.

Overall it seems as if the entrainment of the flies to the light-dark schedule was rather poor, particularly for both sets of wild-type flies. This is evident from the mean activity levels during the simulated 'night' periods, which were not as low proportional to mean activity levels during the simulated 'day' as would be expected. Whilst this should not render the experimental data worthless, it does mean that there is an increased level of noise present in the data and as such analysis is more difficult.

Hypotheses: Following on from previous studies conducted, a number of hypotheses were formulated and tested to inform the modelling efforts made later. These included:

1. Flies with a higher daily mean average activity during entrainment tend to have higher daily mean average activities during free run.
2. Flies that are considered 'noisy' during the entrainment period also tend to produce a greater amount of noise than average at the same time period during the free run.
3. The differences between average activities during entrainment compared to free run are more exaggerated in *tilB* mutants than in Canton S flies.

For all hypotheses testing, the final day of free run data is excluded from the analysis in order to balance the need for as long a free running period as possible for viable analyses to be run with the fact that the activity of all the flies will decrease as the flies grow older.

Hypothesis 1: The daily mean activity of each group of flies during every possible day of entrainment was plotted against the daily mean activity calculated during free run. The results are shown in Figure 2. Appendix V includes graphs displaying this hypothesis tested when only the final three days of entrainment are used for comparison.

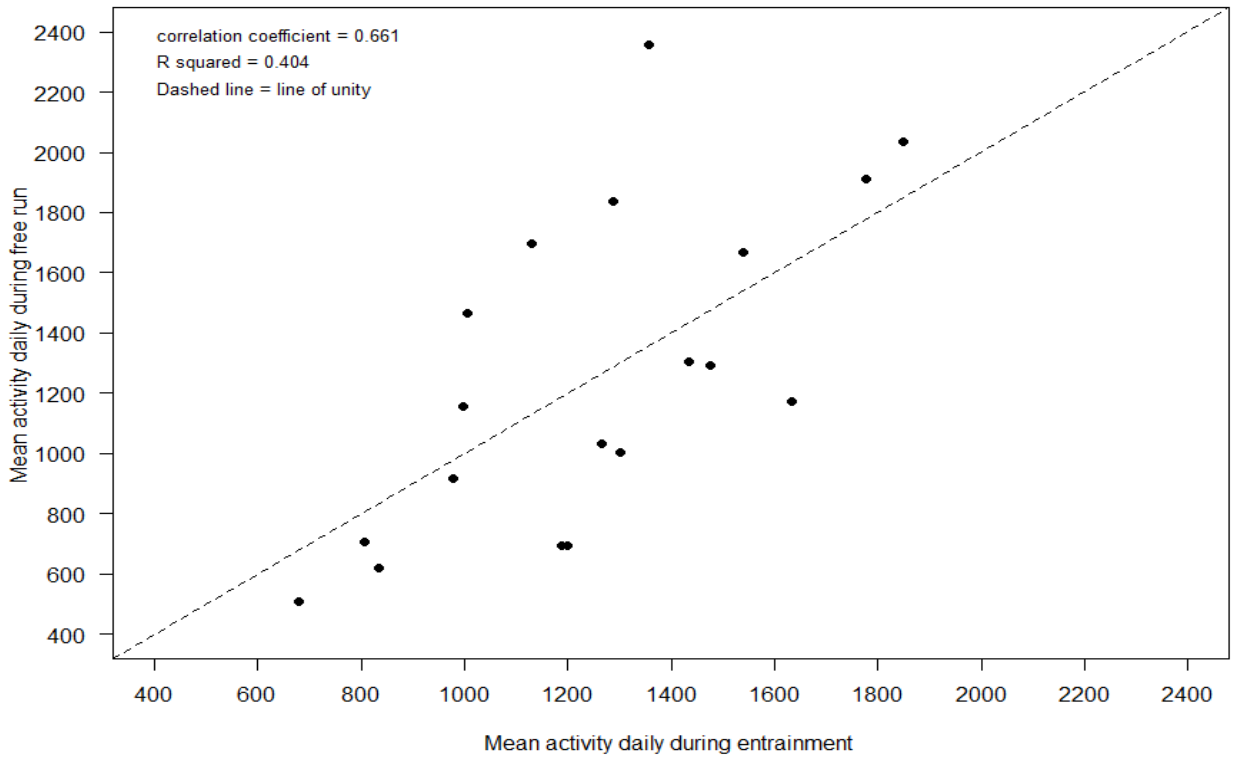


Figure 2 a) Mean activity daily during entrainment against mean activity daily during free run for Canton S flies

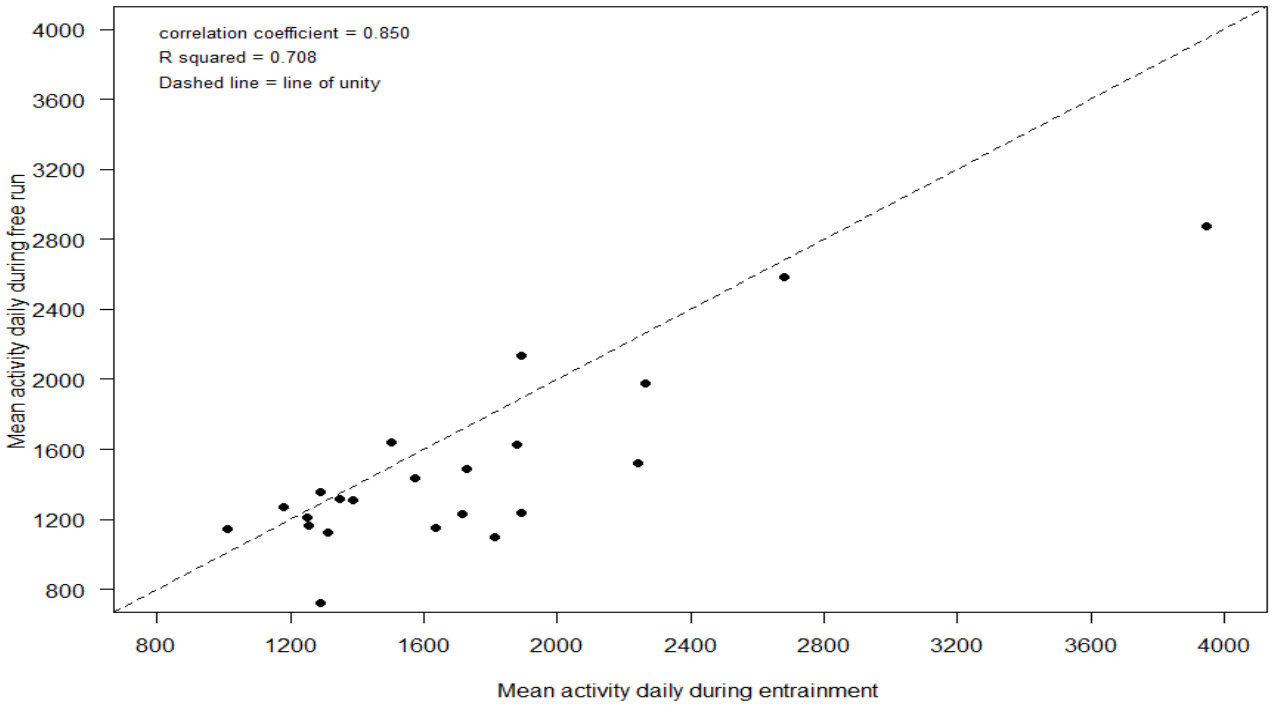


Figure 2 b) Mean activity daily during entrainment against mean activity daily during free run for Canton S flies exposed to pymetrozine

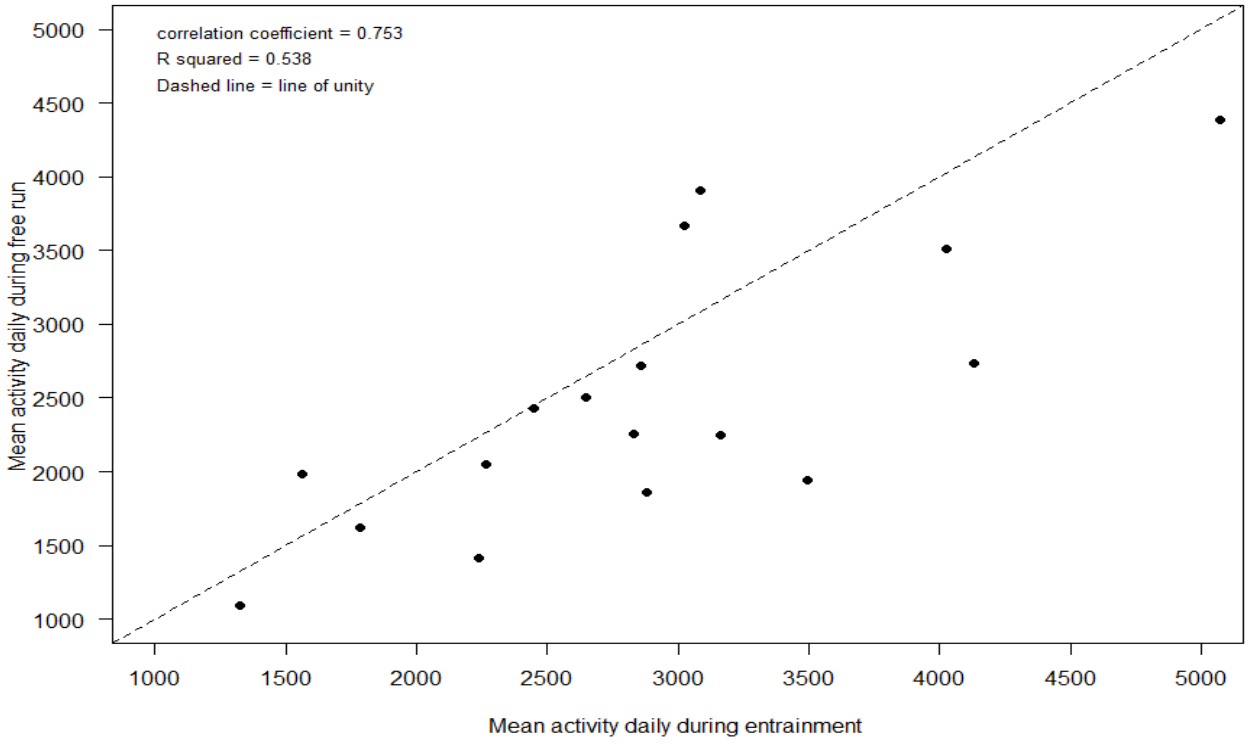


Figure 2 c) Mean activity daily during entrainment against mean activity during free run for tilB flies

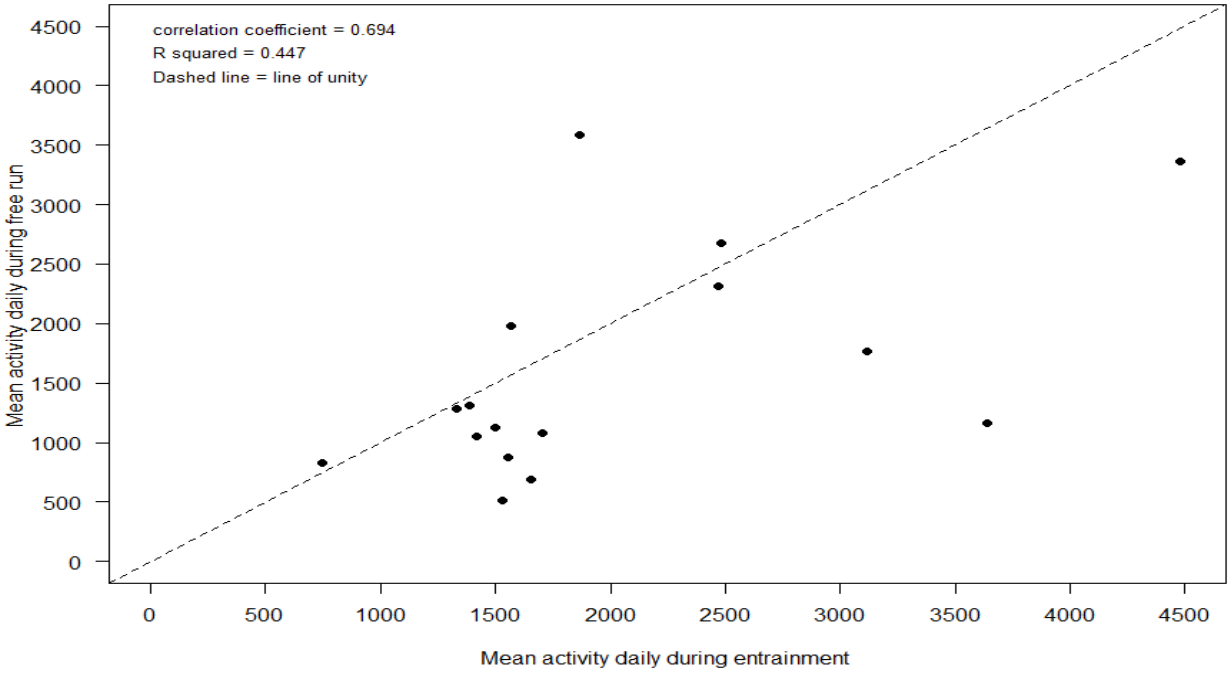


Figure 2 d) Mean daily activity during entrainment against mean daily activity during free run for tilB flies exposed to pymetrozine

Strong correlation coefficients were noted for both Canton S flies exposed to pymetrozine (0.850) and *tilB* flies (0.753), implying that for these fly types mean daily activity during entrainment is a good indicator of mean daily activity during free run. Slightly weaker correlations were found for the other two groups, though stronger correlations have been found previously for Canton S [Ransley, 2013]. As can be seen in appendix V, for all fly types except Canton S that had been exposed to pymetrozine the correlation coefficient increases. This suggests that as the flies tend to equilibrium position (and as such are more entrained by the stimulus) the mean daily activity during free run becomes a better indicator of the same value during free run.

Hypothesis 2: Following the example set by the previous case presentation, the time window investigated for noise was set at between 00:30 and 04:30 (i.e. 30 minutes before the light stimulus was switched on). This was defined as such because all flies should have been asleep at this time. Average activities calculated over this time period in both the entrainment and free run periods were compared. The data range used for comparison was data collected on the final three days of entrainment, which was compared to data collected over the first three days of free run. This was done in an attempt to ensure that transient effects are minimised and that the flies have been exposed to entrainment stimuli for as long as possible and such have reached an equilibrium position.

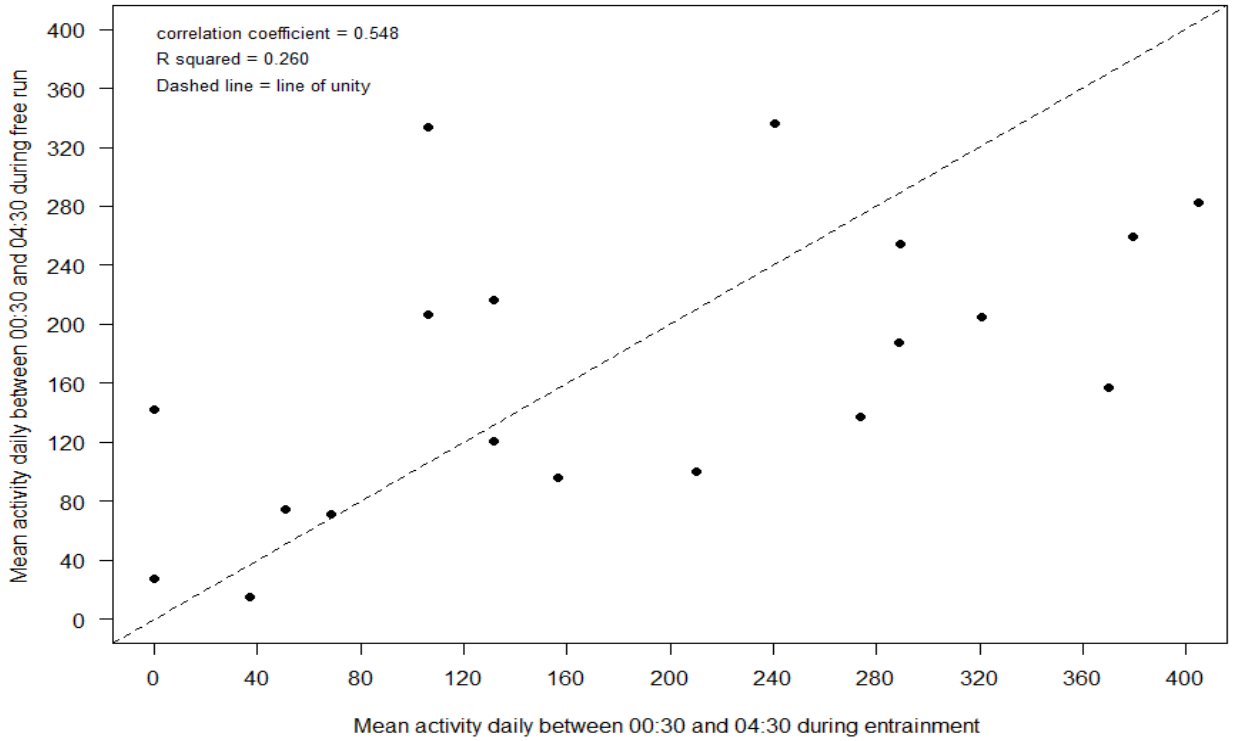


Figure 3 a) Mean activity daily during entrainment against mean activity daily during free run during the 'noise window' for Canton S flies

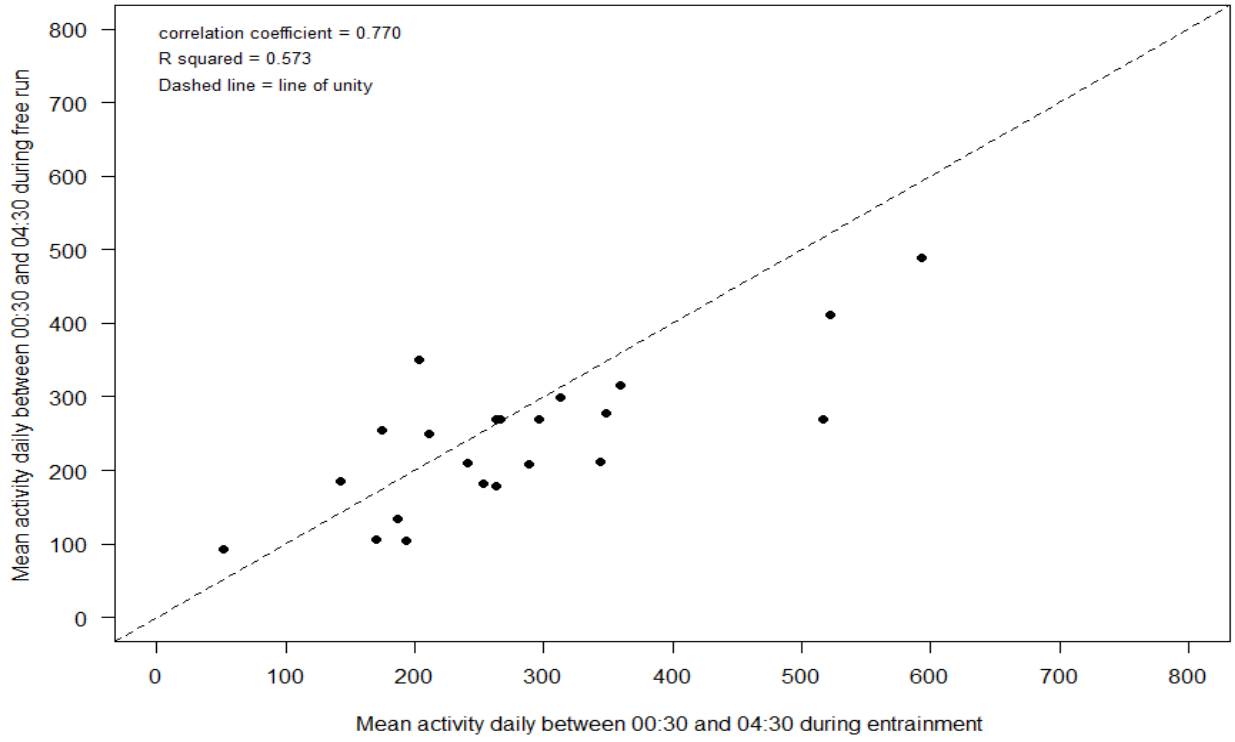


Figure 3 b) Mean activity daily during entrainment against mean activity daily during free run during the 'noise window' for Canton S flies exposed to pymetrozine

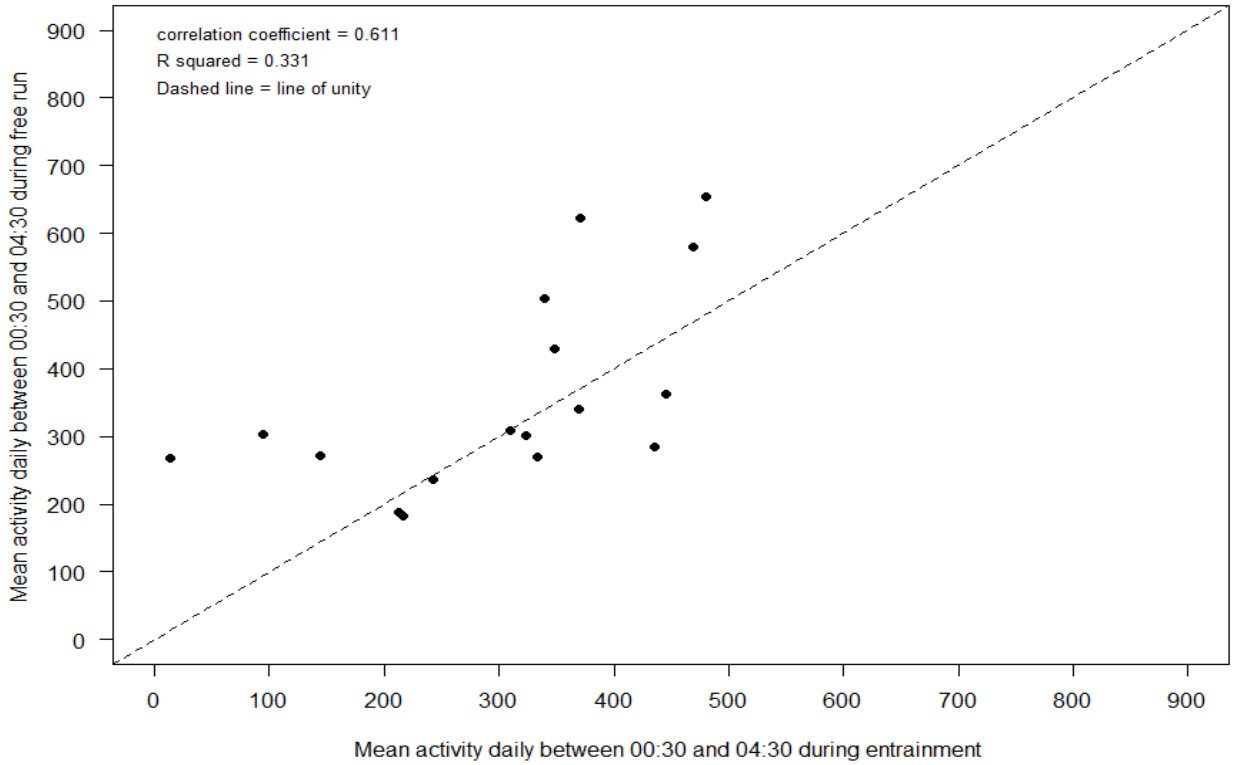


Figure 3 c) Mean activity daily during entrainment against mean activity during free run during the 'noise window' for tilB flies

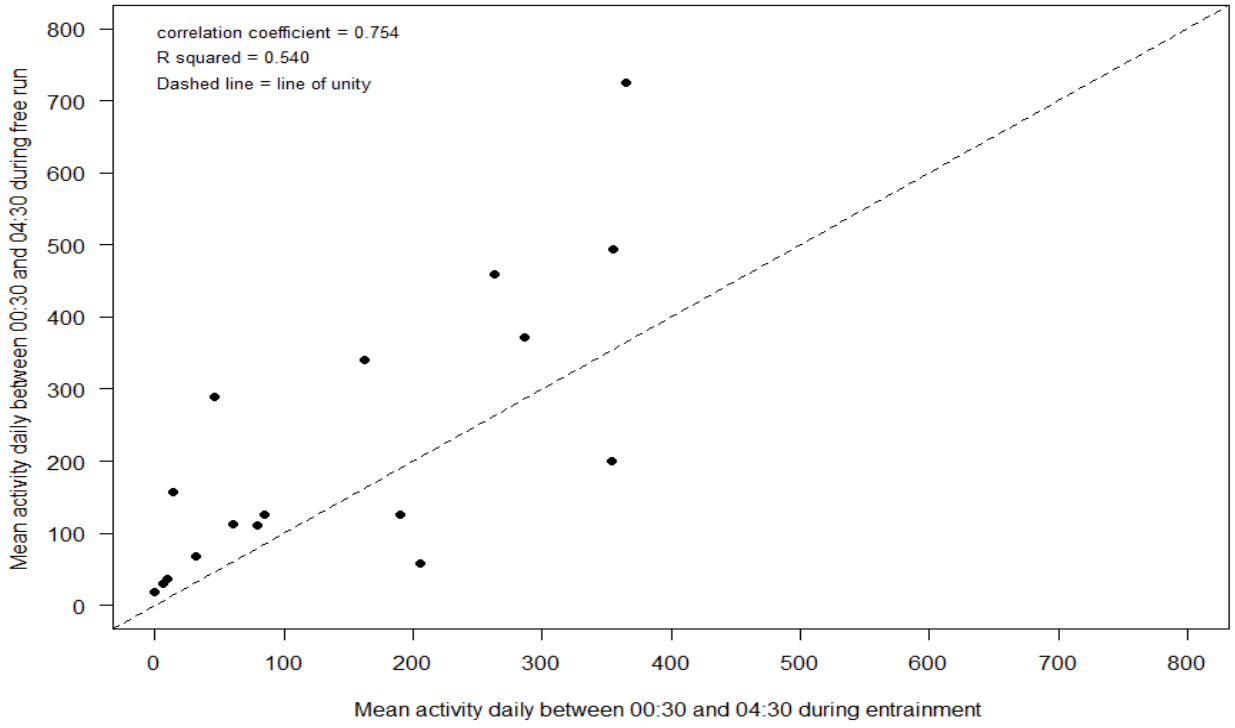


Figure 3 d) Mean activity daily during entrainment against mean activity during free run during the 'noise window' for tilB flies exposed to pymetrozine

In this case the comparisons for both groups of flies that were exposed to pymetrozine showed strong positive correlations, whilst those not exposed had much weaker calculated correlation coefficients, to the extent that Canton S flies showed essentially no correlation (in contrast to earlier results). This strongly suggests that, at least for the two fly groups exposed to the insecticide, the level of noise displayed during the entrainment period is a good indicator of noise levels during the free run.

Hypothesis 3: In order to investigate this final hypothesis, the mean activity values for the final three days of entrainment and the first three days of free run for each half hour were calculated. Figure 4 below shows the absolute net difference between mean activity during entrainment and during free run for both Canton S and *tilB* groups, with all flies which survived the entire experiment included in the analysis. Positive values indicate a higher mean activity during entrainment than free fun at that time period, and vice versa.

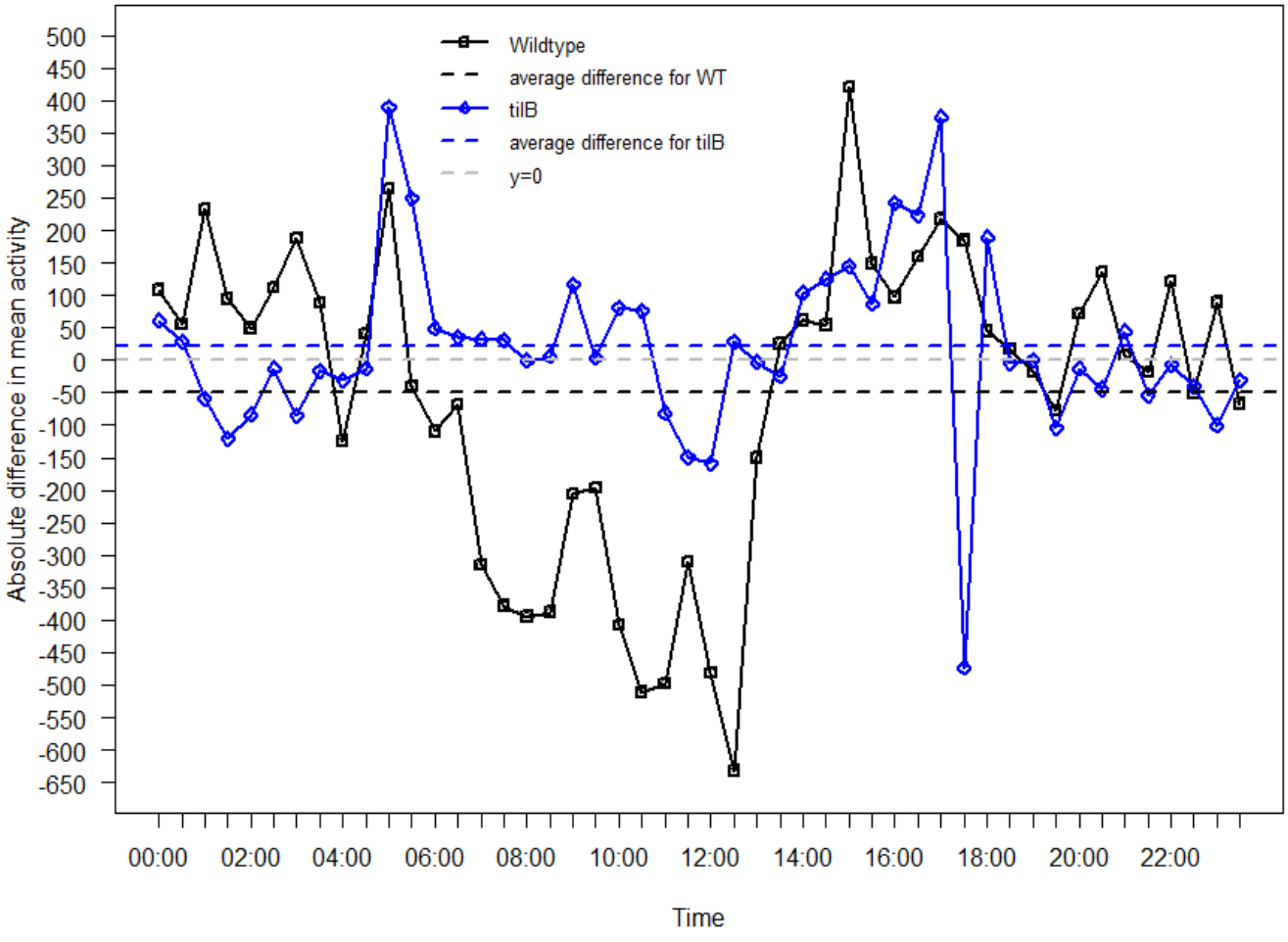


Figure 4) Time against absolute difference in mean activity for both Canton S and tilB flies. The average absolute difference for both genotypes, along with the line y = 0, are included for reference

As can be seen above, there does not appear to be much of a relationship between Canton S and *tilB* flies when looking at the absolute differences average activity during entrainment and free run at different times.

There are some similarities between the two groups however: both groups show a huge spike in activity difference 05:00, the time point of light onset during entrainment, and seem to have similar difference values from 20:00 to 04:00 (corresponding to the dark period of entrainment).

There are two clear distinctions between the genotypes based off this figure – *tilB* flies seem to exhibit a much greater activity shortly after 17:00 during the free run than during the entrainment period, which is not visible for wild type flies) whilst on the other hand wild type flies seem to be much less active during entrainment during the hours that approximately correspond to a light stimuli being present than during the corresponding hours of free fun (which is not seen for *tilB* flies). This second point could be considered the result of masking, whereby the light stimulus leads to the fly being seemingly paralysed for a length of time, but the length of time for which this large difference in activity values occurs suggests that this is not the case.

Both fly groups have overall average differences very close to zero, which suggests that the average activity over a day is fairly constant regardless of the presence, or lack of, stimuli. This could have an ecological background in terms of attempting to minimise energy loss whilst also maximising efficiency. Further experiments should be done investigating this before any conclusions can be drawn.

5. Modelling background

Some of the previous attempts at modelling fly activity have constructed models using Poisson functions, with the rationale that the mean and variance can sometimes be similar when looked at over an entire day, and the type of count data we are working with can lend itself well to being fitted to a Poisson distribution.

Unfortunately the level of overdispersion present in the data collected during the experiment suggested that this may not be the right avenue to explore. Figure 5 plots the mean against the variance for first a single, then all, Canton S flies. The variance to mean ratio, a standard measure for overdispersion, was typically found to be at least 20 for the wild type flies and was even greater for the other fly groups (*tilB* flies in particular tended to have very large values). Given this, it was decided to instead attempt to fit a negative binomial distribution to the data sets, with the hope that the additional free parameter with respect to a Poisson distribution would allow for a better fit. The modelling was focused on reproducing behaviour in Canton S flies during entrainment, and to look at the effect of noise on activity levels, whilst also investigating the effect of noise on the activity level of a fly during the free running period.

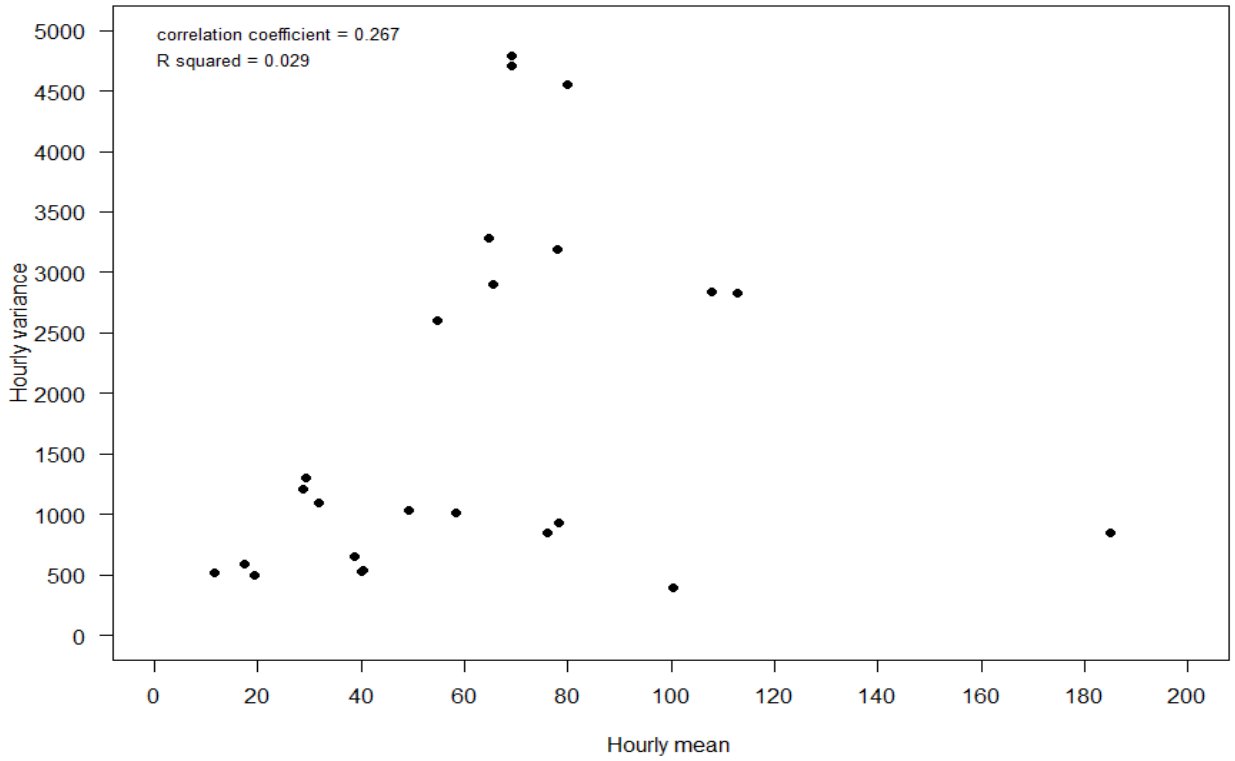


Figure 5 a) Hourly mean against hourly variance for a randomly selected Canton S fly

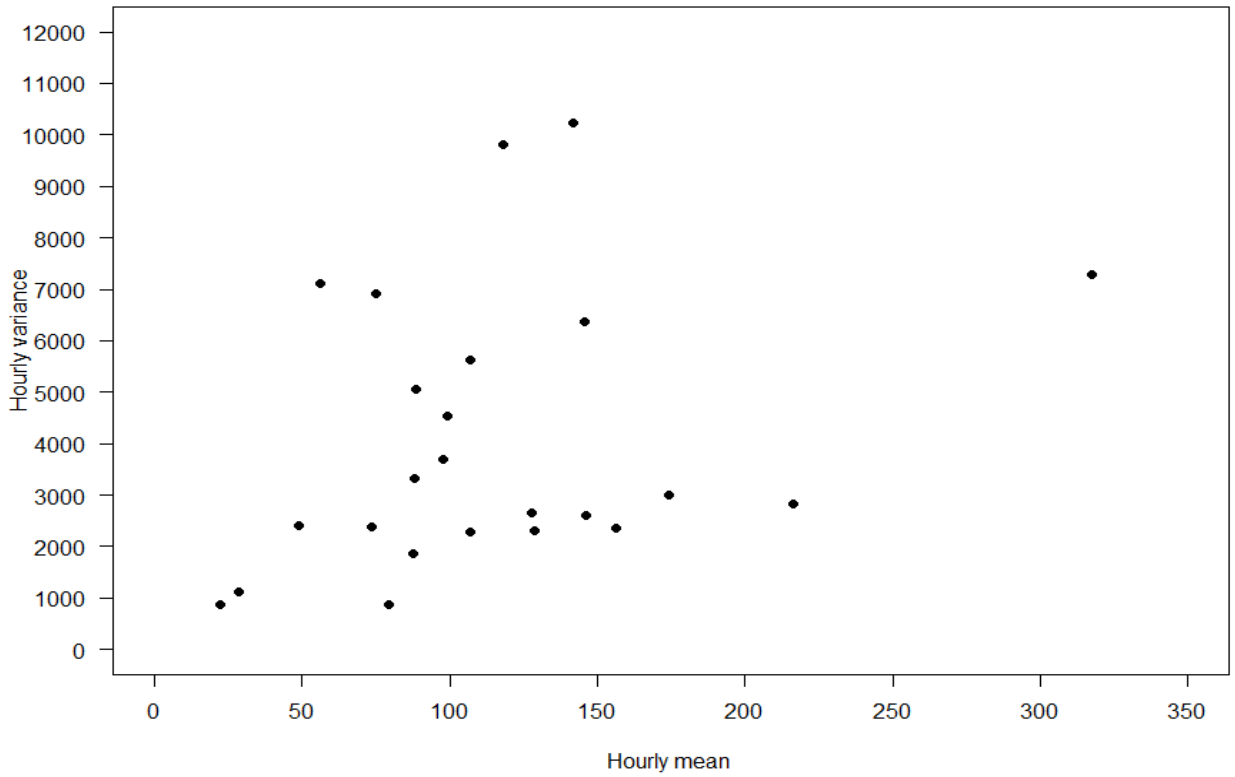


Figure 5 b) Hourly mean against hourly variance for all Canton S flies

A model described in a previous case presentation was adapted for this purpose:

$$Act_t = f(e^{-At}(NB(r, p) + \alpha N(0,1)))$$
$$p = \max(\sin(\omega_t), 0)$$
$$\omega_t = \frac{2\pi}{48} t(1 - \delta(\phi(Act_{t-15t-1}) - \mu))$$

Here activity at t is modelled by a negative binomial distribution with *p* being the probability of success and *r* being the number of predefined failures. A stochastic term *N*, drawn from a normal distribution with mean equal to zero and variance equal to one and multiplied by a constant α, is included to model the large amount of noise present in data, and an exponentially decaying term was also included with exponent A to account for the reduction in fly activity over time that is the result of ageing. This value was then converted by the function f into an integer by rounding, because activity at any time point cannot be a non-integer.

The probability of success was estimated as being the maximum value of a sinusoidal function (because of the cyclic nature of activity), with phase equal to ω_t. The phase term itself was equal to a constant multiplied by the time interval t, coupled with a term that allows for present activity to be modified by previous activity. This term includes the weighted average activity over the previous fifteen time periods, with coefficient δ, minus μ, which was set as the mean daily activity divided by the number of intervals per day. Negative values of the sinusoidal function were set to zero.

6. Estimation of parameters and model output

The model was fitted using maximum likelihood packages in R. Here the parameters that need to be estimated were r, A, δ and α. It was hoped that hypothesis 2 might provide the means to estimate δ without recourse to maximum likelihood estimation, but the poor correlation observed between the noise periods in the separate time series means that this is out of the question. In addition to this it is not possible to use values calculated in the past by similar methods because of the change in distribution from a Poisson to a negative binomial. Possible relationships between activity levels at various times throughout entrainment and the 'noise window' in the first three days of the experiment were investigated (see appendix VI) but were not found.

Maximum likelihood estimation for negative binomial distributions is well documented in textbooks and the codes of the relevant packages on R were first checked to ensure that they correctly followed the general procedure. Rather than ignore the first 24 hours of data (as is usually done to avoid complications that can occur with using data from that time period), all data from the entrainment period was used so that the noise inherent in first day activity (from there being no proper desirable entrainment, new environmental conditions, vibrational fluctuations from being moved etc.) could be looked at. Data from the free running period was used only to inform the data generated for the free run.

Figure 6 below consists of two average actograms; both used starting values taken from separate, randomly selected Canton S flies. Neither one of them is a close match for any individual fly, nor do they show a particularly rigid entrainment pattern as there are spells of activity in the dark period of entrainment. This is most likely due to weak entrainment in the flies that provided these values however.

The log-likelihood value was significantly higher for a negative binomial model than for a Poisson model (with values of-18590.3 as compared to -25756.7 for the first fly fitted and -15271 compared to -29638 for the second), indicating a much better comparative model fit.

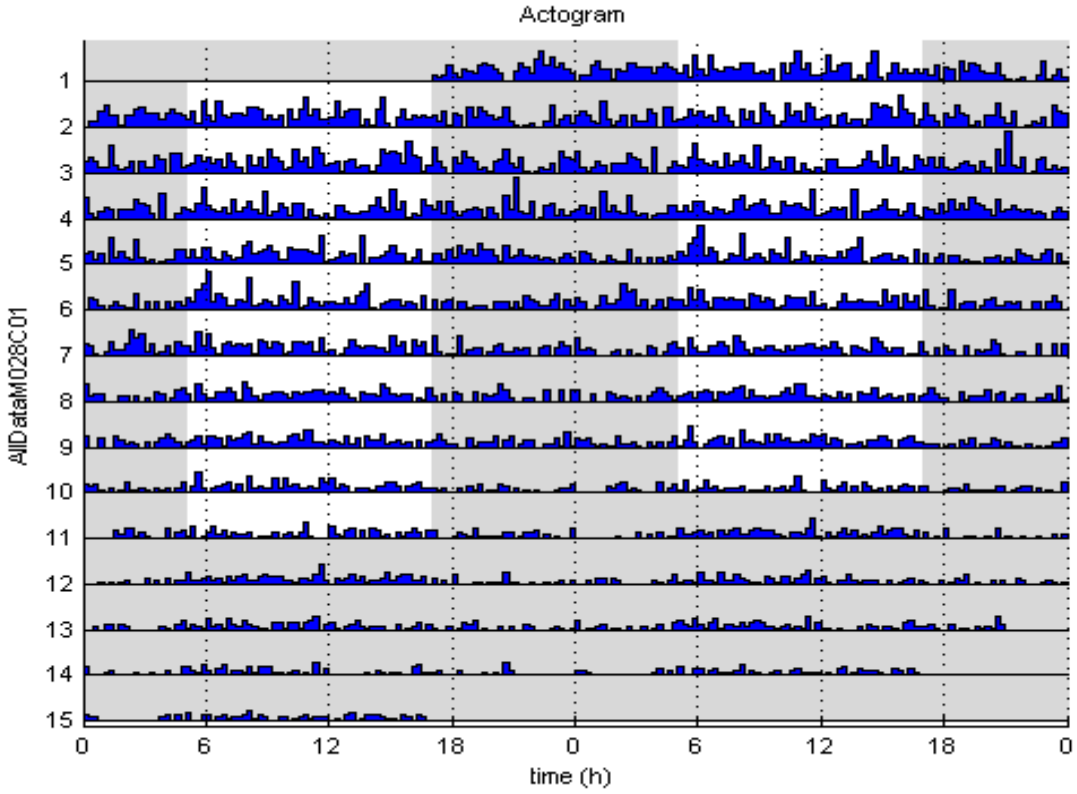


Figure 6 a) An actogram generated which simulates Canton S fly activity over the entire course of the experiment. The parameters used included A=0.0005, δ = 5 and α = 0.02.

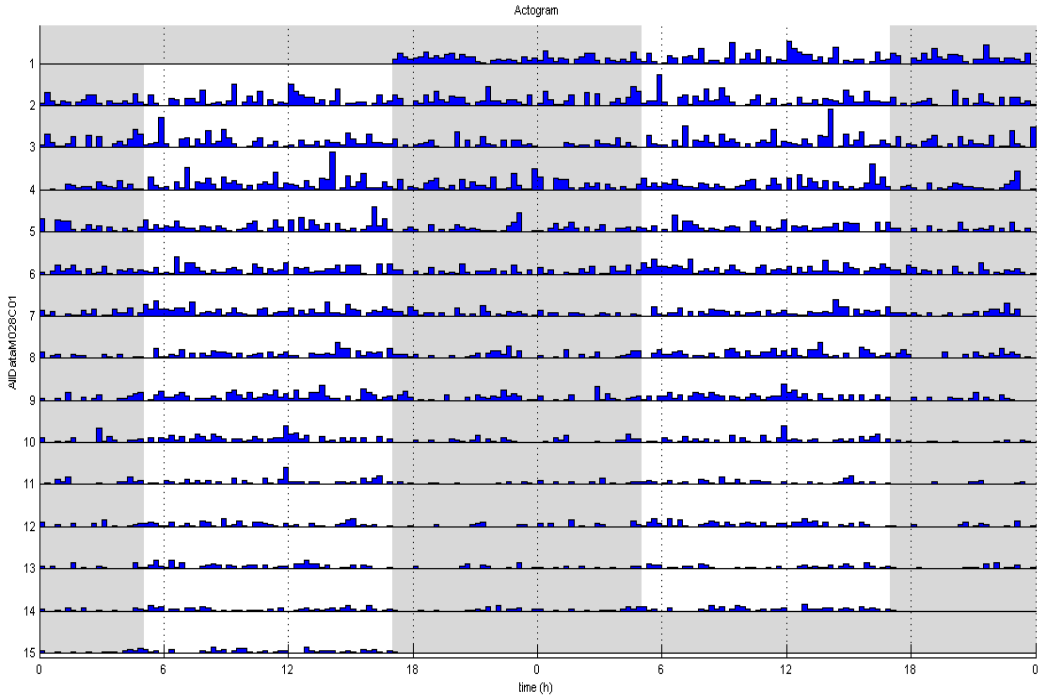


Figure 6 b) Another actogram generated with different initial conditions which simulates Canton S fly activity over the entire course of the experiment. The parameters used included A=0.0005, δ = 3 and α = 0.03.

7. Discussion

As was mentioned in an earlier section, the entrainment was not as robust as one might have hoped. This could be the result of dim background light during the dark periods of entrainment (despite the precautions taken against this), which would thus lead to an increase in mean activity during these time intervals. Whilst it could be argued that the data sets are more realistic as a result, as in the wild the flies will never be in complete darkness, in practice this lack of robustness complicates the analysis as greater noise levels are present (which is particularly problematic considering that a central project aim was to look at noise). To prevent this from occurring again in the future equipment such as incubators should be used to completely block out any unwanted sources of light.

The use of a negative binomial distribution is common when dealing with overdispersion in other topics i.e. helminth overdispersion in human hosts. Future models of activity should strongly consider using the distribution as a basis to build upon. The concept of activity during previous time periods have a strong influence on current potential activity should be studied in greater detail then here, where only the previous 15 time periods were considered – it should be possible to extend or shorten this as necessary.

The models themselves generated noisy data that seemed plausible, but the problems intrinsic in the data set itself mean that realistically it was difficult to simulate living flies accurately. Repeating the experiment with a more robust entrainment pattern would be ideal, as would possibly extending the models to cover other fly strains such as *tilB* mutants.

The activity monitors used in the experiment shone just a single beam down the centre of each tube. This means that any fly activity that occurs in another part of the tube is not recorded, potentially leading to an underestimation of overall activity. In order therefore to collect more accurate data in future experiments Trikinetics MB5 multi-beam detectors should be used instead, as whilst these monitors can only monitor 16 flies at any one time they are able to shine 17 infra-red beams (which are equally spaced along the tubes containing the flies) at once. This would permit a much more detailed analysis of each fly's behaviour.

Future experiments could include another fly genotype in the form of *per01* mutants which lack a circadian clock but retain full use of their chordotonal organs. Running a similar experimental design and including *per* mutants, some of which had been exposed to pymetrozine, could therefore be of great interest as a comparative measure.

It could also be useful to inject the pymetrozine into the selected flies rather than exposing them to the insecticide via doped food. This would guarantee that each fly receives a significant concentration of pymetrozine and so decreases the probability of individuals not feeding enough to be greatly affected. This would be especially helpful since pymetrozine is considered to be an 'all or nothing' drug [Ausborn *et al.*, 2005] and the only current method available of checking the impact of the feeding is via the destructive test outlined in appendix II. Thus a non-destructive method of ascertaining the sensory capabilities of a fly exposed to the drug would be ideal.

Overall it would seem that whilst the model used has some advantages, it needs tuning and improving, and further experiments are needed in order to study this topic in more depth.

Acknowledgements

I would like to thank both of my supervisors for their help and guidance. I would also like to thank Ryan Kavlie and Nerissa Marziano for their assistance and advice with regards to setting up and running the experiment, and Lucy Firth for providing the details on preparing the feeding assays containing pymetrozine.

References

Albert, J.T., Nadrowski, B., Kamikouchi, A. and Gopfert, M.C. (2006). 'Mechanical tracing of protein function in the *Drosophila* ear' Protocol Exchange (2006) doi:10.1038/nprot.2006.364

Simoni, A., Wolfgang, W., Stanewski, R., and Albert, J. (Submission) 'A mechanosensory input pathway to the Drosophila circadian clock' Currently in the submission process

Ashworth, W. (2012) 'The role of behaviour in temperature entrainment of the Drosophila circadian clock' UCL CoMPLEX Summer Project: 2012

Ausborn, J., Wolf, H., Mader, W. and Kayser, H. (2005) 'The insecticide pymetrozine selectively affects chordotonal mechanoreceptors' The Journal of Experimental Biology 208, 4451-4466 doi:10.1242/jeb.01917

Glossop, N. 'Circadian timekeeping in *Drosophila Melanogaster* and *Mus Musculus*' Bio- chemical Society Essays Biochem. 2011 49, 19-35; doi:10.1042/BSE0490049

Harrewijn, P. and Kayser, H. (1997). 'Pymetrozine, a Fast-Acting and Selective Inhibitor of Aphid Feeding. *In-situ* Studies with Electronic Monitoring of Feeding Behaviour' Pesticide Science (1997) Vol. 49(2) doi: 10.1002

Hart, G. (2013) 'How sugar tunes the clock' Cell Metabolism, Vol. 17(2) 155-156, 5 February 2013

Hughes, A.T.L. And Piggins, H.D. (2012) 'The Neurobiology of Circadian Timing: Chapter 18' Editor: Kalsbeek, A.

Kavlie, R., Kernan, M. and Eberl, D. (2010) 'Hearing in Drosophila Requires TilB, a Conserved Protein Associated With Ciliary Motility' Genetics Society of America doi:10.1534/genetics.110.114009

Libert, S., Bonkowski, M.S., Pointer, K., Plecher, S.D. and Guarente, L. (2012) 'Deviation of innate circadian period from 24 h reduces longevity in mice ' Aging Cell, Vol. 11(5): 794-800 (2012)

Milhiet, V., Etain, B., Boudebesse, C. and Bellivier, F. 'Circadian biomarkers, circadian genes and bipolar disorders J Physiol Paris. 2011 Dec;105(4-6):183-9. doi: 10.1016

Ouyang, Y., Andersson, C.R., Kondo, T., Golden, S. and Johnson, C.H. (1998) 'Resonating circadian clocks enhance fitness in cyanobacteria' Proc. Natl. Acad. Sci. USA Vol. 95, pp. 8660–8664, July 1998

Piggins, H.D. and Guilding, C. (2011). 'The neural circadian system of mammals' Essays Biochem. (2011) 49, (1–17)

Ransley, M. (2013) 'Behavioural Influence On Circadian Patterns in *Drosophila melanogaster*: A Modelling Approach' UCL CoMPLEX Case Presentation 1

Risau-Gusman, S. and Gleiser, P.L. 'Modelling the effect of phosphorylation on the circadian clock of Drosophila' Journal of theoretical Biology Vol 307:53-61 doi.org/10.1016/j.jtbi.2012.05.003

Sechser, B., Reber, B. and Bourgeouis, R. (2002) 'Pymetrozine: Selectivity spectrum to beneficial arthropods and fitness for integrated pest management' J.Pest Sci. 75: 72-77

Sehadova, H., Glaser, F.T., Gentile, C., Simoni, A., Giesecke, A., Albert, J. and Stanewsky, R. (2009) 'Temperature Entrainment of *Drosophila*'s Circadian Clock Involves the Gene *nocte* and Signaling from Peripheral Sensory Tissues to the Brain' *Neuron* Vol. 64(2) 251-266

Appendices

Appendix I: Pymetrozine doped feeding assay preparation

Following from the standard format 1.5 ml of sugar agar solution was placed into 20 separate feeding assays. To make this solution, 1.5g of agar was added to 150ml of water. This was then heated in a microwave for approximately two minutes so that the agar melted completely. 7.5 grammes of sugar (in this case, glucose was used) was then added, and the mixture was vibrated to allow the sugar to dissolve totally. The desired amount of solution was then put into the assays and allowed to dry and cool down.

The pymetrozine was then dissolved in Dimethyl sulfoxide (DMSO) at a pymetrozine level of 1000 parts per million, which equates to the use of 1ml of DMSO for every microgram of pymetrozine used. 20 μl of this solution was then used to coat the surface of each assay, and allowed to dry. In total 60 flies were placed into the pymetrozine doped feeding assays, in groups of ten per assay, and were left for a day before being removed from the assays and placed on regular food sources for two days before the experiment began.

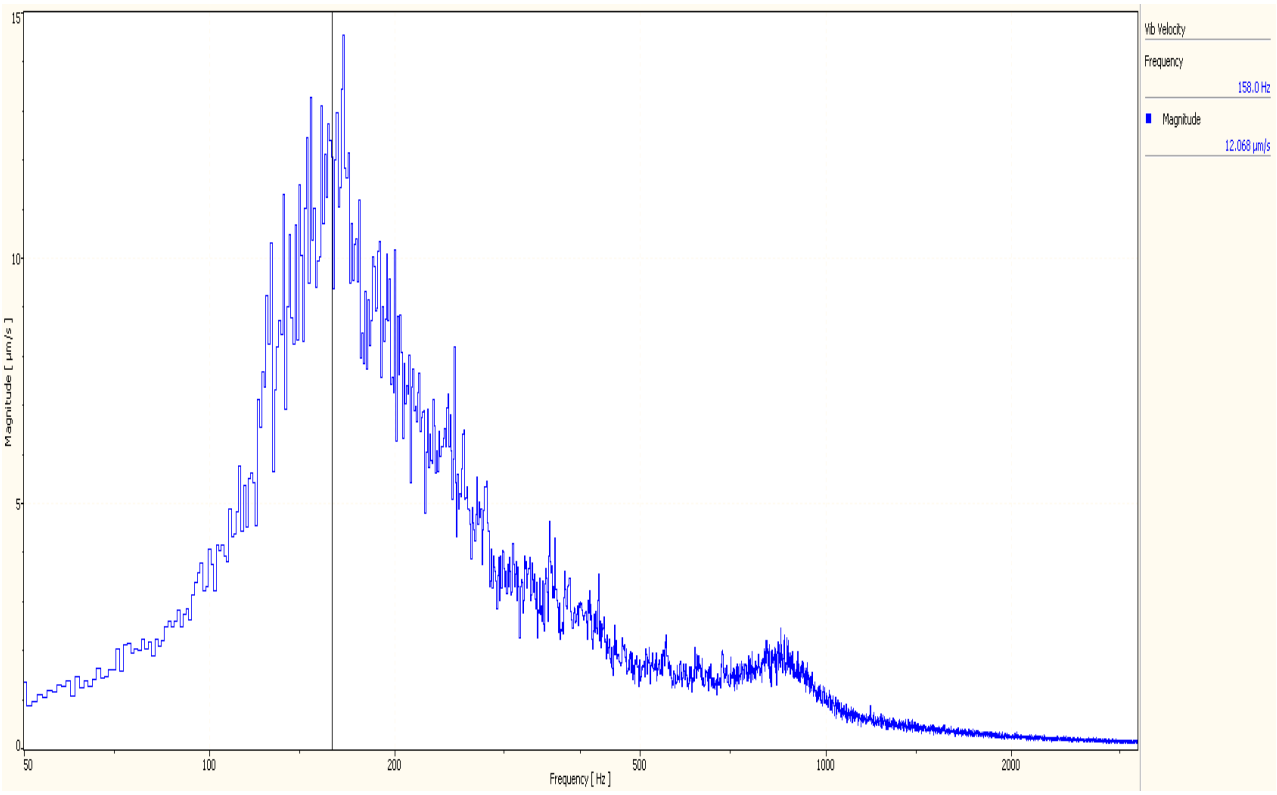
Appendix II: Mechanical response measurement protocol and results

A complete description of the experimental protocol is provided by Albert *et al.* in the 2006 paper 'Mechanical tracing of protein function in the *Drosophila* ear'. In short, the underlying rationale behind these measurements is that the auditory neurons of the flies being measured are able to amplify the small vibrations provided by the equipment. This feedback is designed to help the ear with regards to weak sounds.

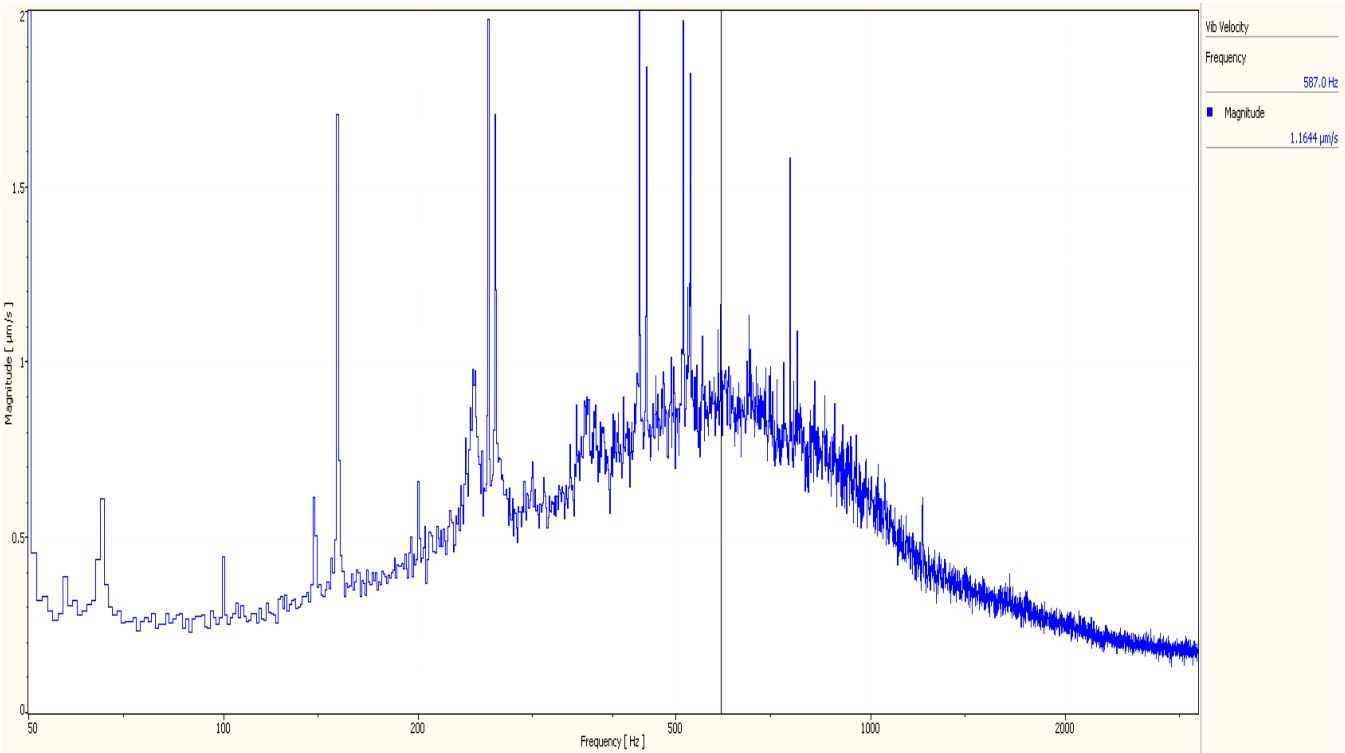
The mechanics of the receiver are characteristic of the fly type being investigated. For example for a Canton S fly a value of between 150 to 350 Hz is expected for the resonance frequency, whilst a much higher value of around 700 Hz can be found for *tilB* mutants (which have a much more passive arista). Further than this, the Compound Action Potentials can also be investigated, as they should not be present in *tilB* flies.

Measurements were taken from flies belonging to each one of the four groups and are presented below. The measurements were taken with the help of Nerissa Marziano. It is clear from the figures that whilst Canton S flies that had been fed pymetrozine did not demonstrate complete deafness, as the *tilB* mutants do, they suffer from at least some level of hearing loss when compared to regular Canton S individuals. This is indicated by the increase in the resonance frequency and the weakness of the compound action potentials found. This severe ablation of function indicates that the drug feeding had some effect on the animals hearing. On the other hand, there appears to be no difference in the resonance frequency of *TilB* flies that had been put on pymetrozine doped feeding assays and regular *tilB* mutants – this is hardly surprising considering that *tilB* mutants are unable to amplify stimulus induced vibrations of their antenna and are entirely deaf.

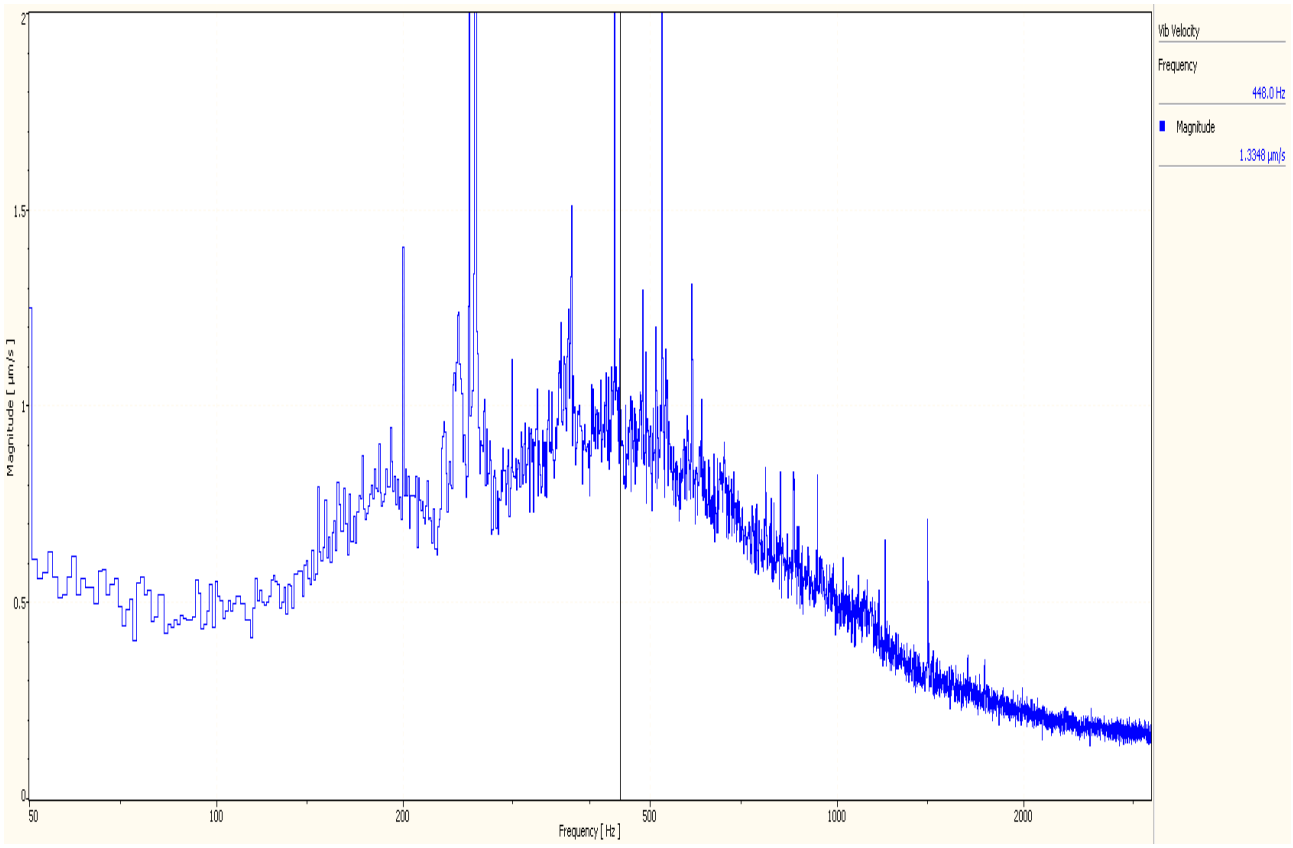
As it has been previously noted [Harrewijn and Kayser, 1997] that aphids have been able to recover hearing function over time if the initial pymetrozine concentration was sufficiently low enough, more recordings were made on Canton S flies that had been feeding on regular food for over a week after being exposed to pymetrozine for one day. The flies that were underwent the measurement procedure displayed resonance frequencies in a similar range to those that had been tested after being placed on regular food for only two days (see below).



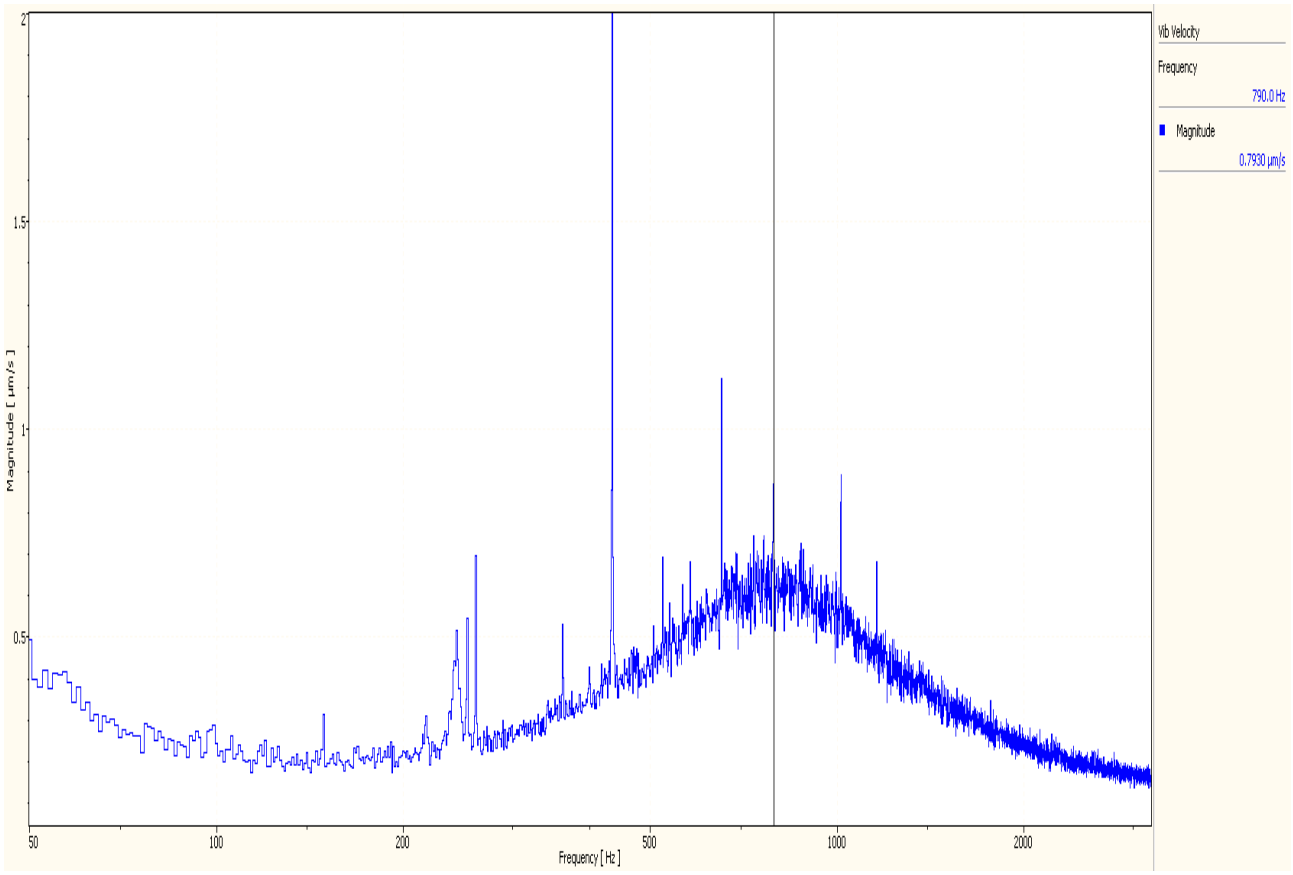
Frequency against magnitude for a Canton S fly that had not been exposed to pymetrozine



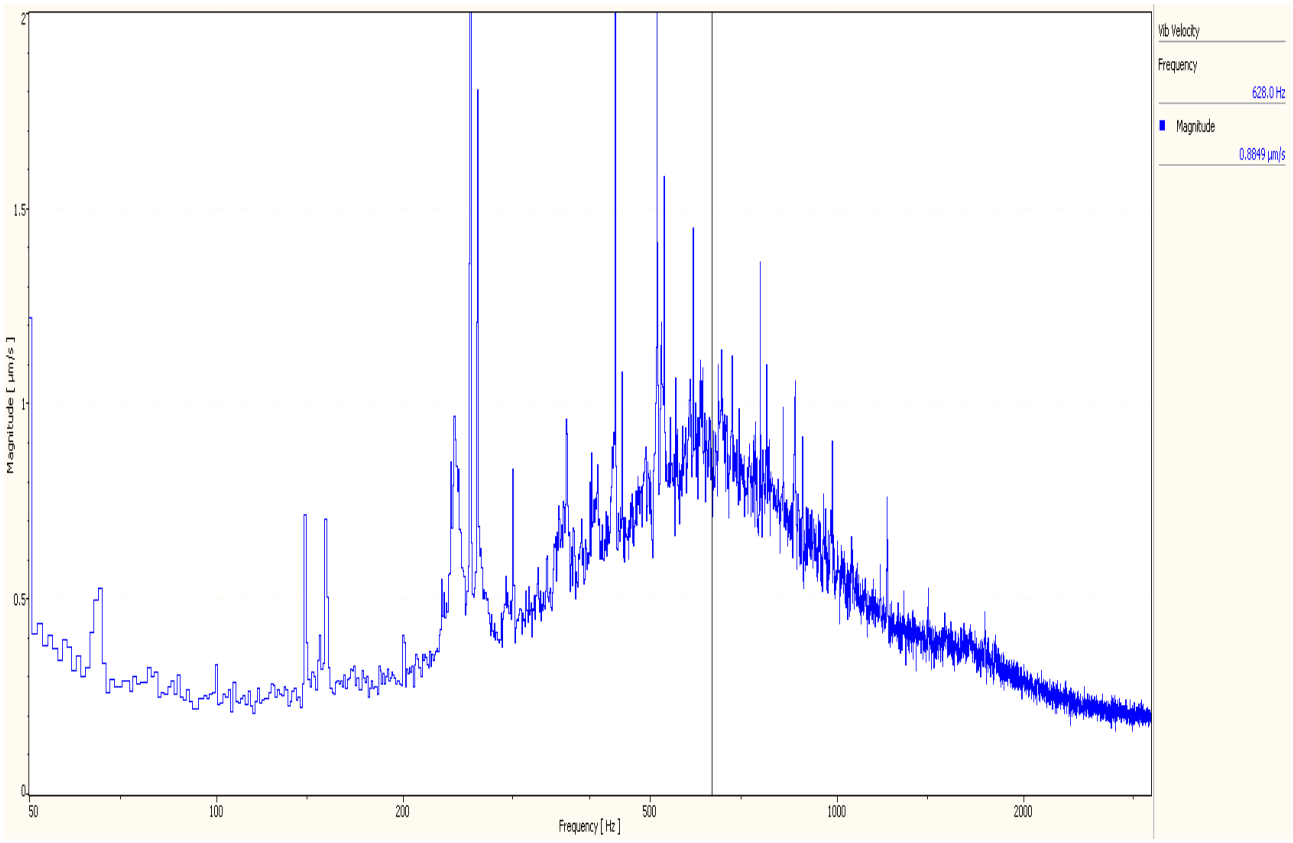
Fluctuations for a Canton S fly two days after being removed from being exposed to pymetrozine for one dat



Fluctuations for a Canton S fly that had been removed from one day of exposure to pymetrozine seven days before recording

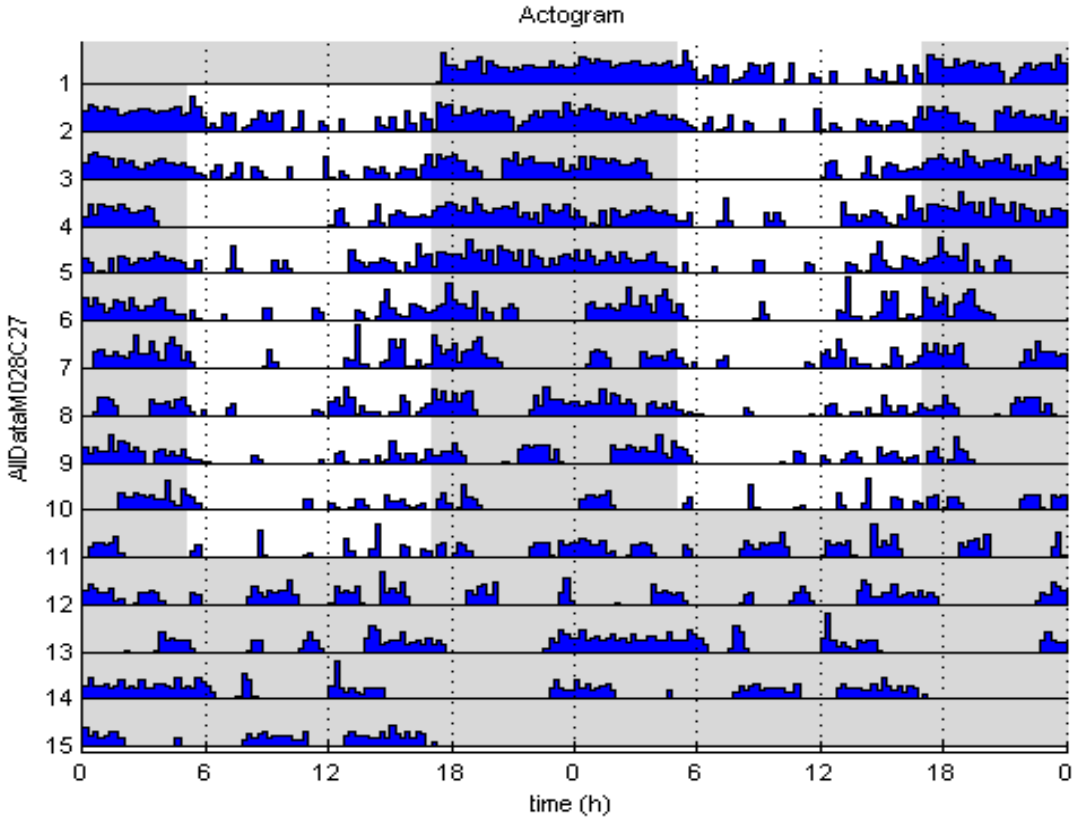


Fluctuations for a tilB fly that had not been exposed to pymetrozine

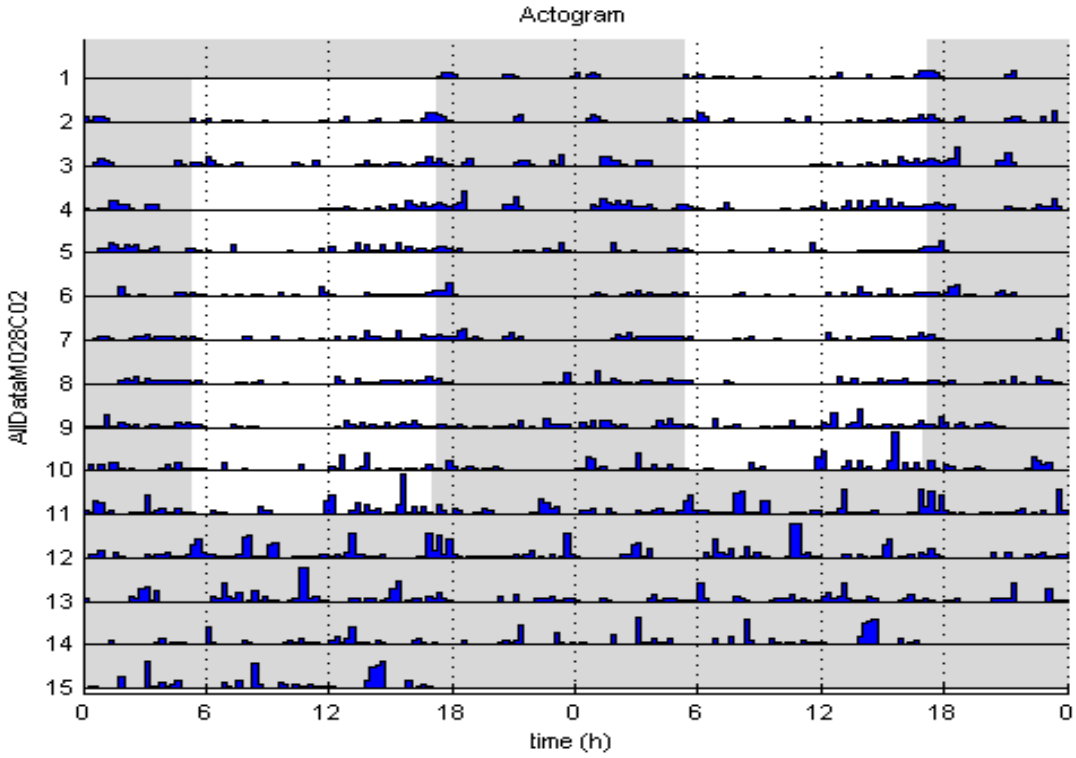


Fluctuations for a fly that had been removed from one day of exposure to pymetrozine two before the recording

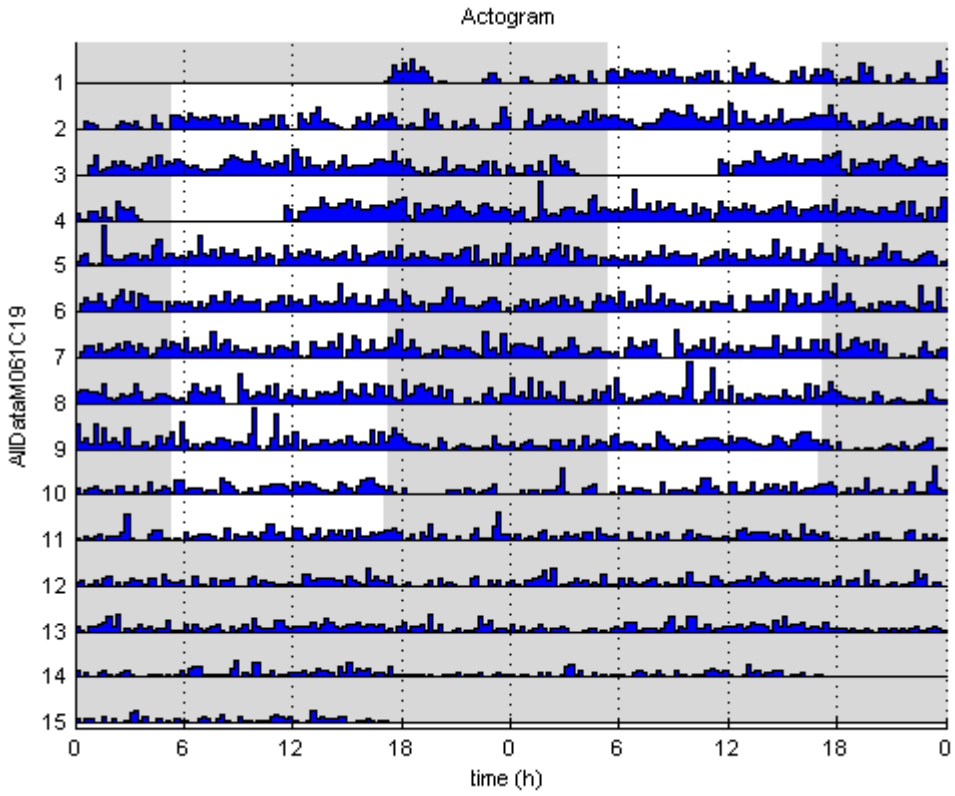
Appendix III: Actograms for randomly chosen flies from each group



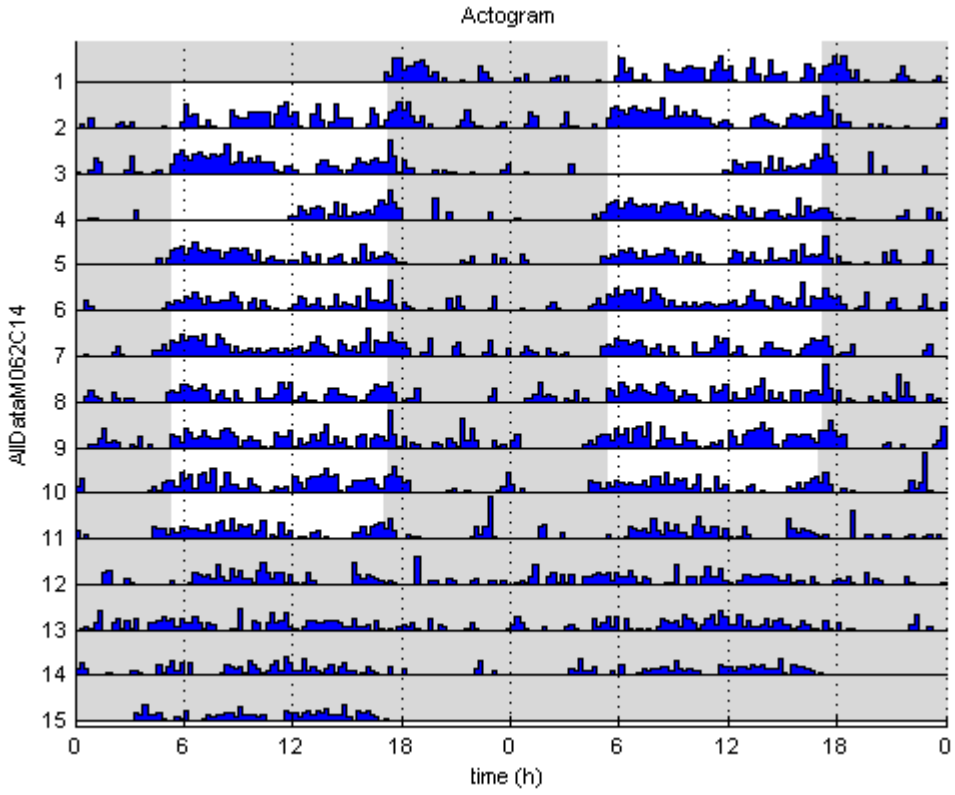
This Canton S fly displays large amounts of activity during the dark periods of entrainment, which then decreases over time



This Canton S fly that has been exposed to pymetrozine exhibits sparse, low level activity during entrainment, but becomes more active during free run

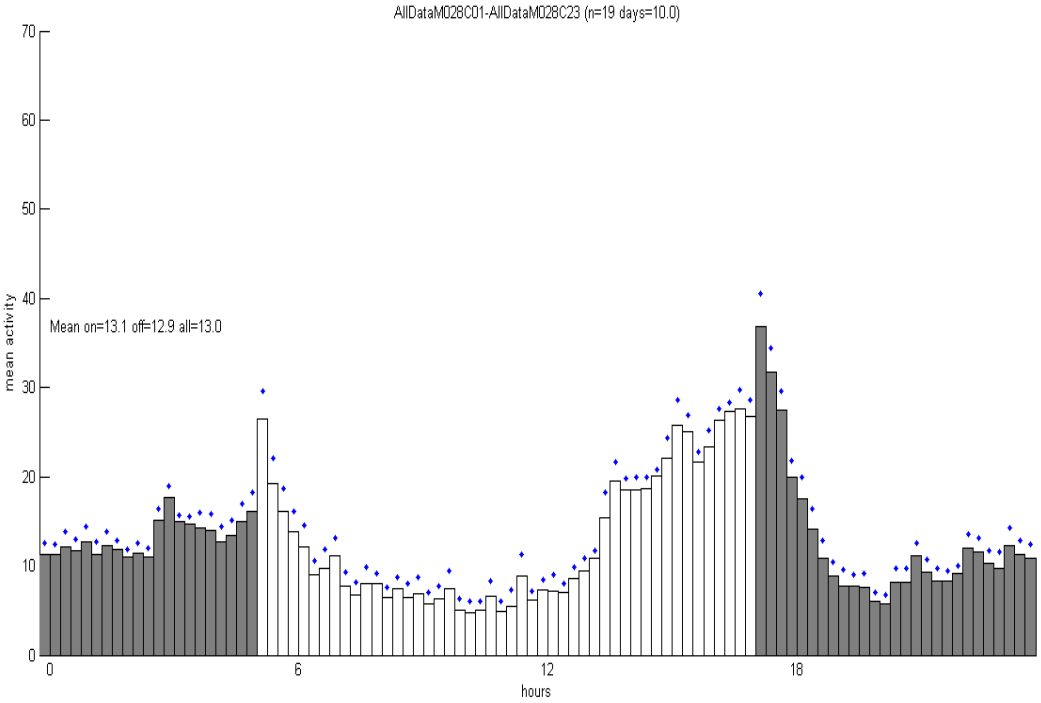


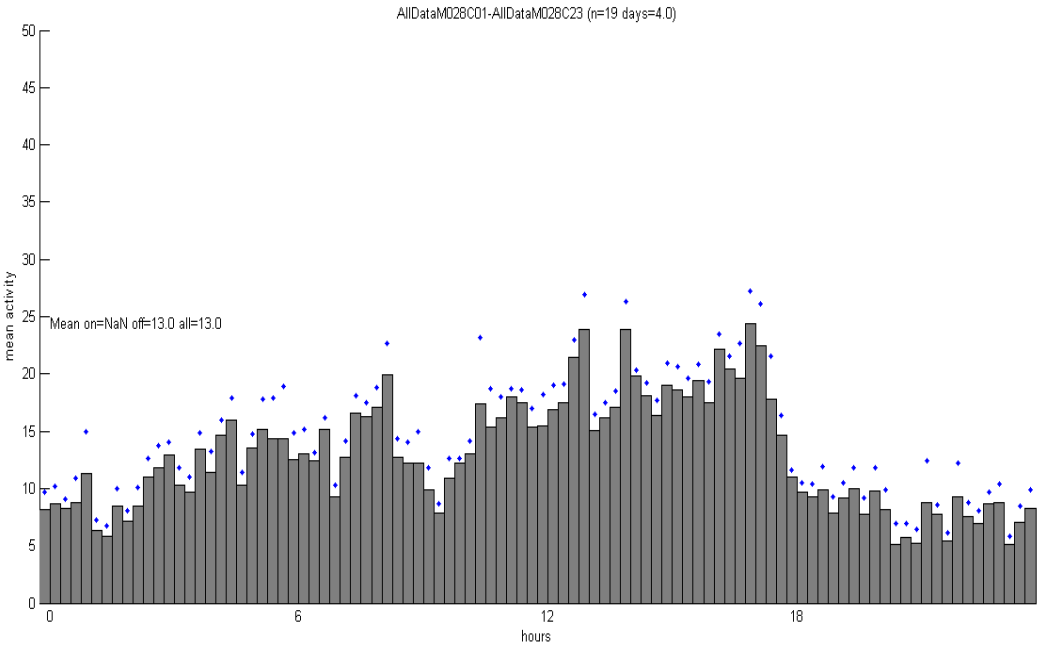
This tilB fly shows a general decrease in activity level as time goes on



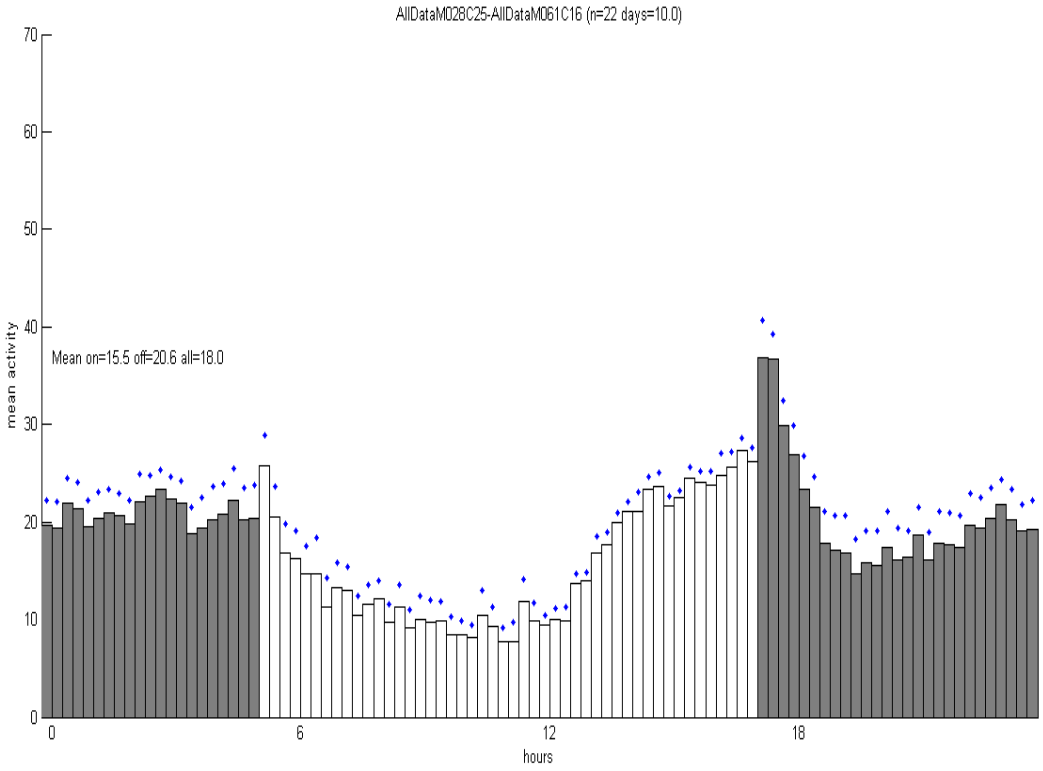
This tilB fly that has been exposed to pymetrozine has a much greater activity in light periods of entrainment than dark periods, with decreased activity in later days

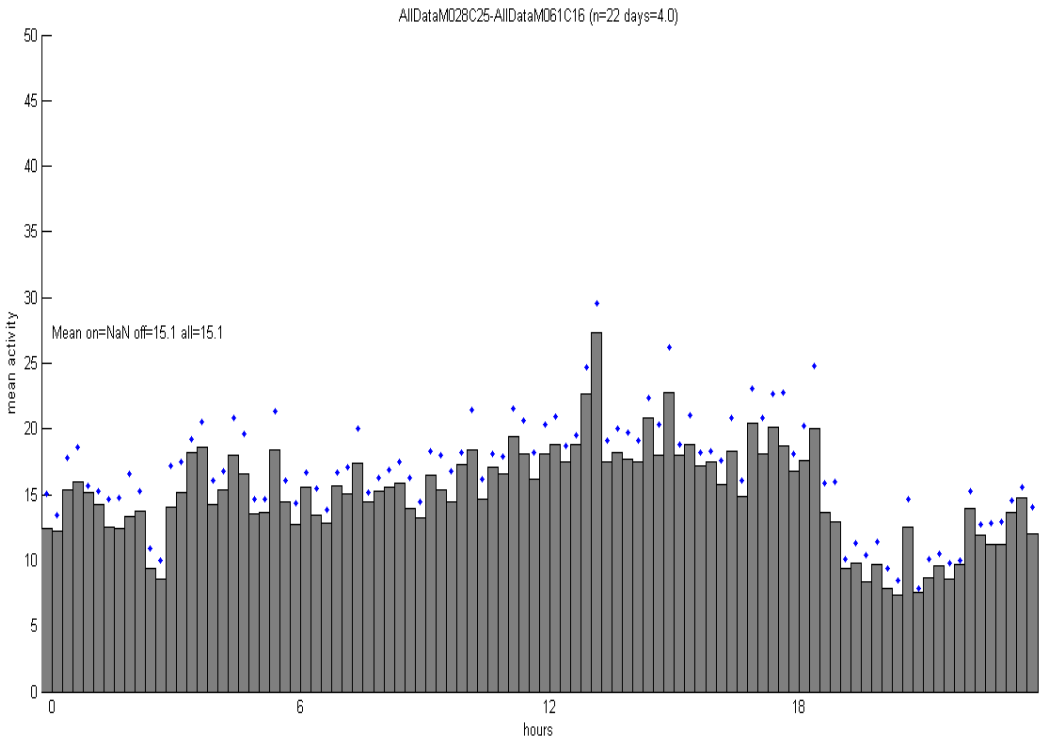
Appendix IV: Histograms and bar charts for different fly groups



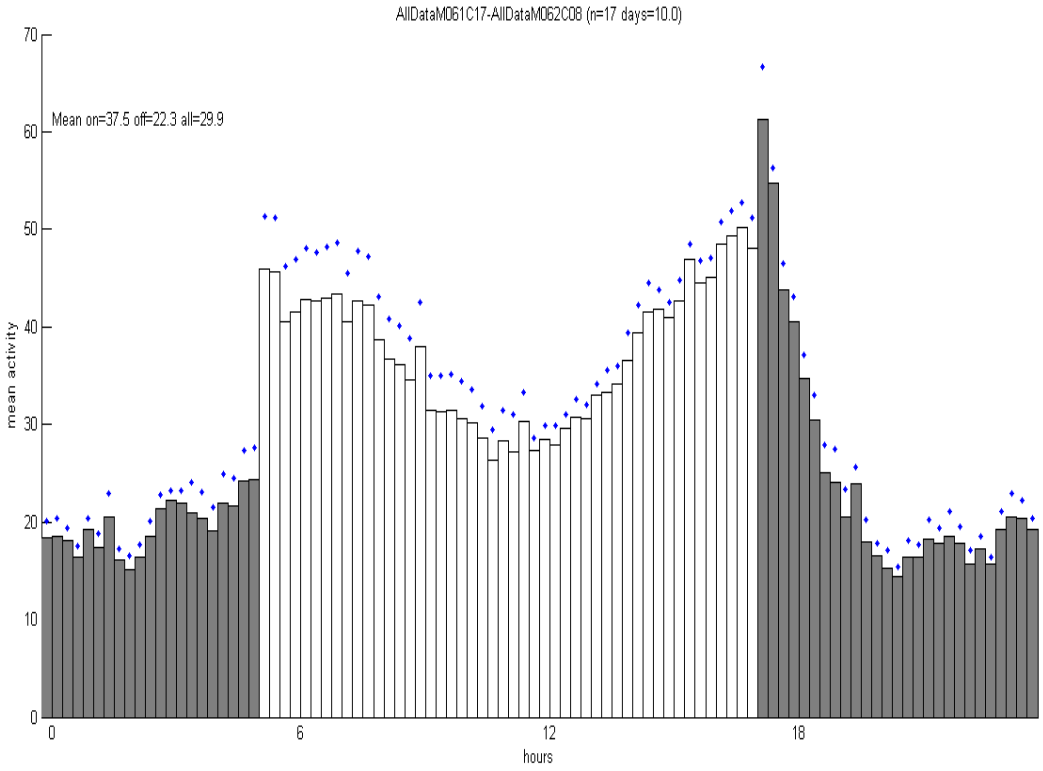


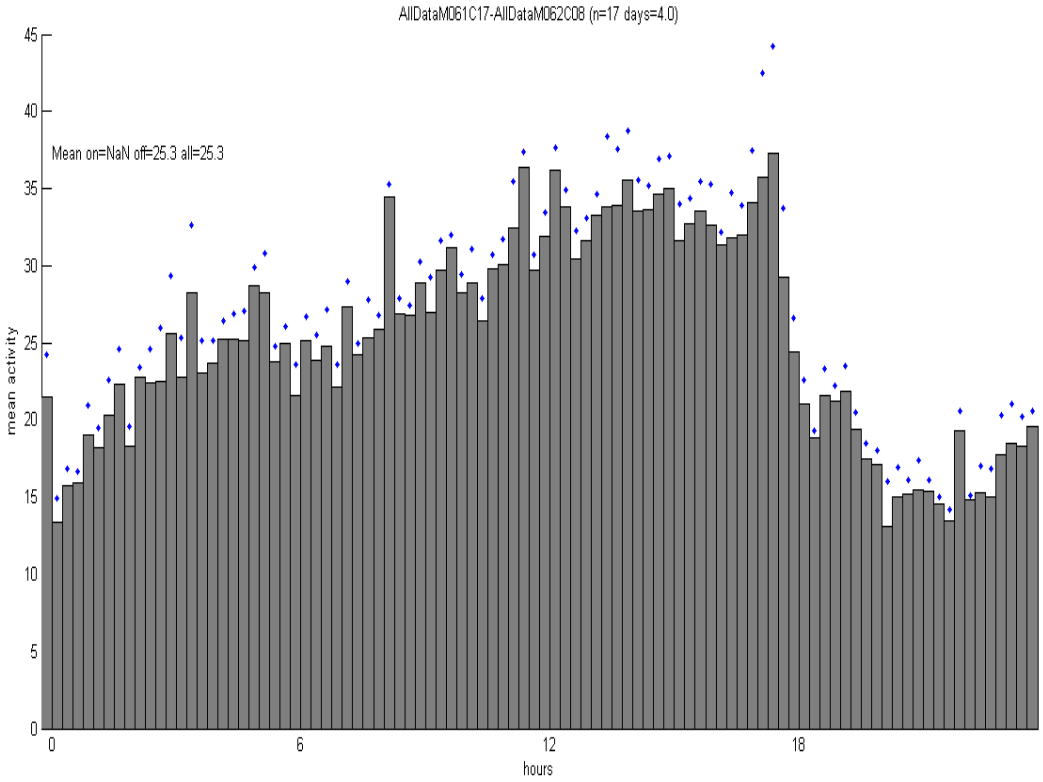
Histograms for Canton S flies, with the entrainment period on top and the free run underneath – light bars indicate times during entrainment with the light stimulus switched on. The entrainment histogram somewhat displays the expected diurnal pattern of activity, which is missing from the free run histogram.



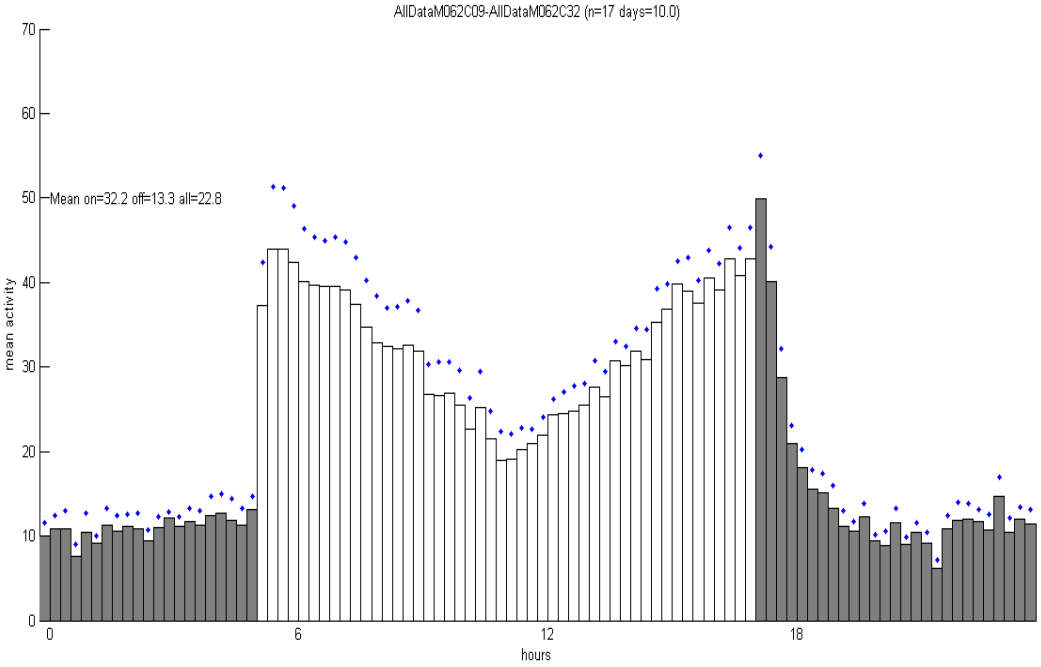


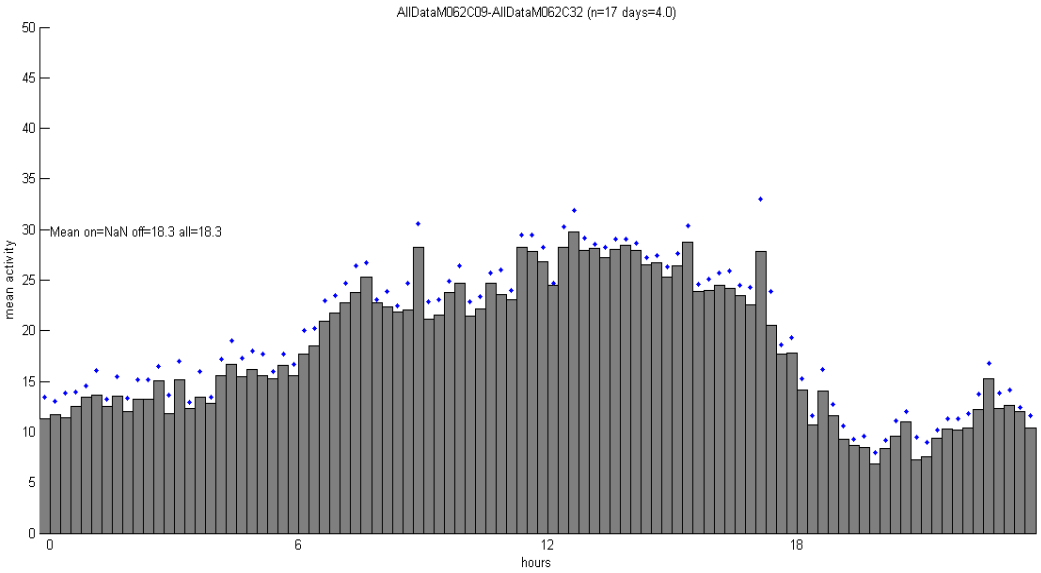
Histograms for Canton S flies that have been exposed to pymetrozine, with the entrainment period on top and the free run underneath. The entrainment histogram barely displays the expected diurnal pattern of activity, which is missing from the free run histogram, though the peak in anticipatory activity seems to be less in this case.



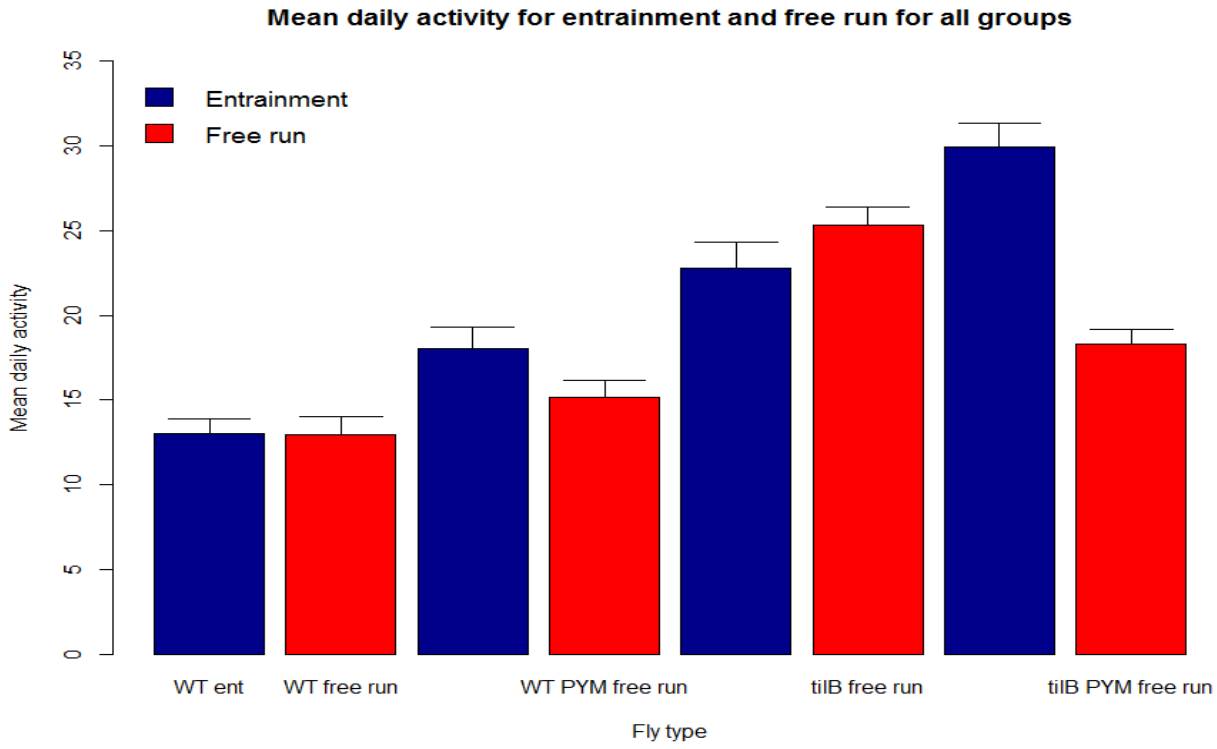


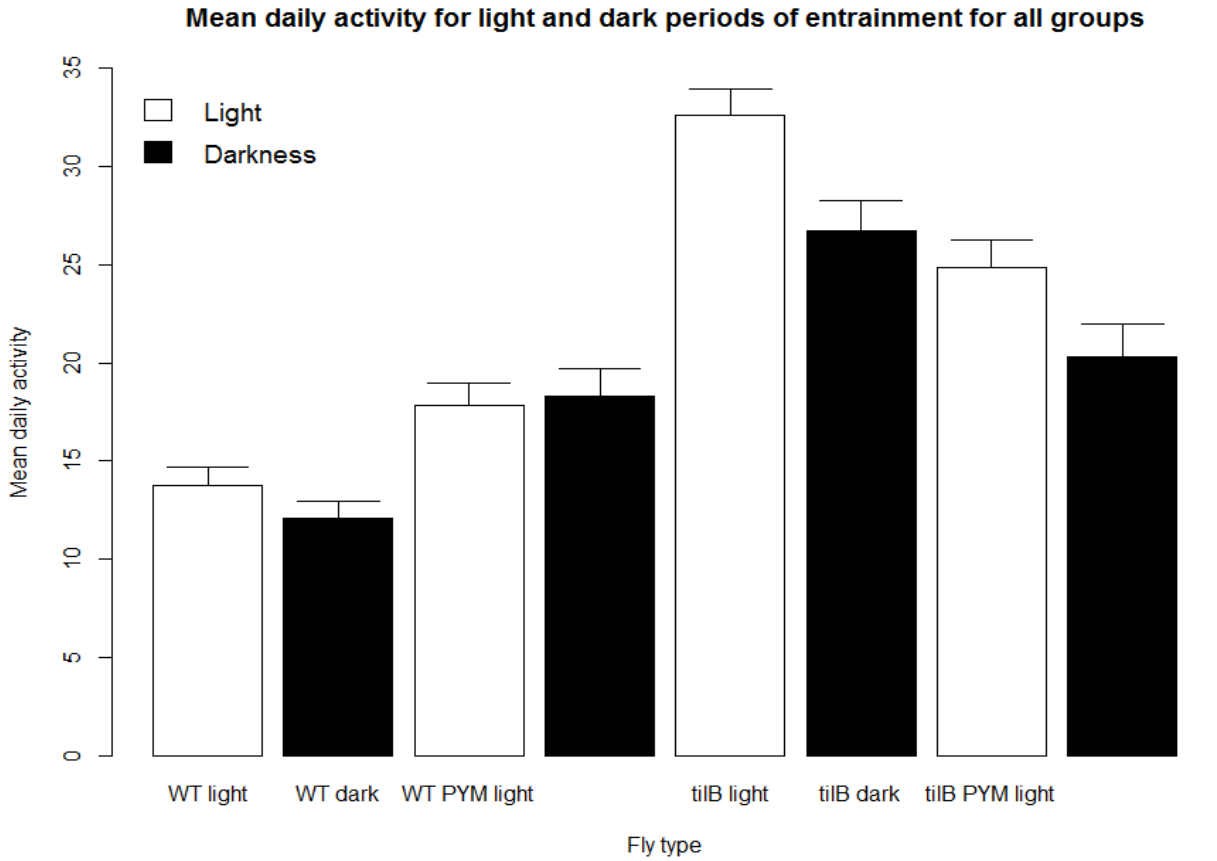
Histograms for *tilB* flies, with the entrainment period on top and the free run underneath. The entrainment histogram displays some of the expected features of a diurnal pattern of activity, which is missing from the free run histogram. *TilB* flies seem to be much more active than wild type flies.





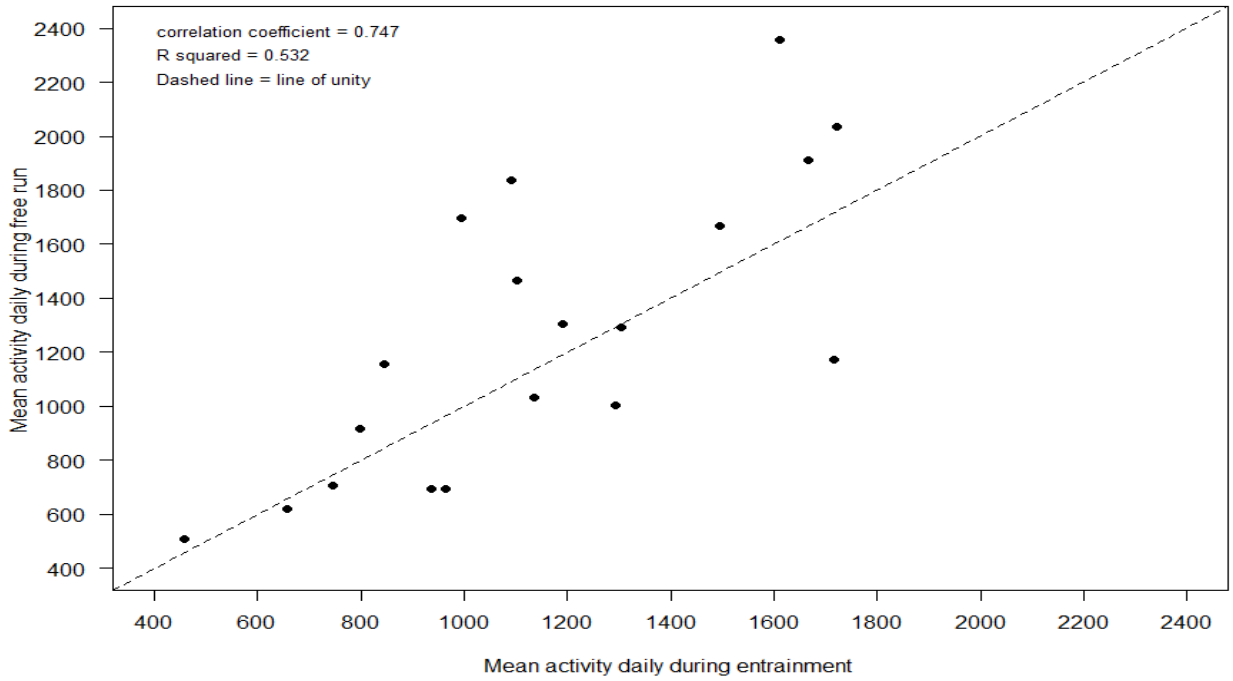
Histograms for *tilB* flies that have been exposed to pymetrozine, with the entrainment period on top and the free run underneath. The entrainment histogram displays the expected diurnal pattern of activity, which is missing from the free run histogram, with incredibly sharp increases in activity at the switch over times between light and dark.



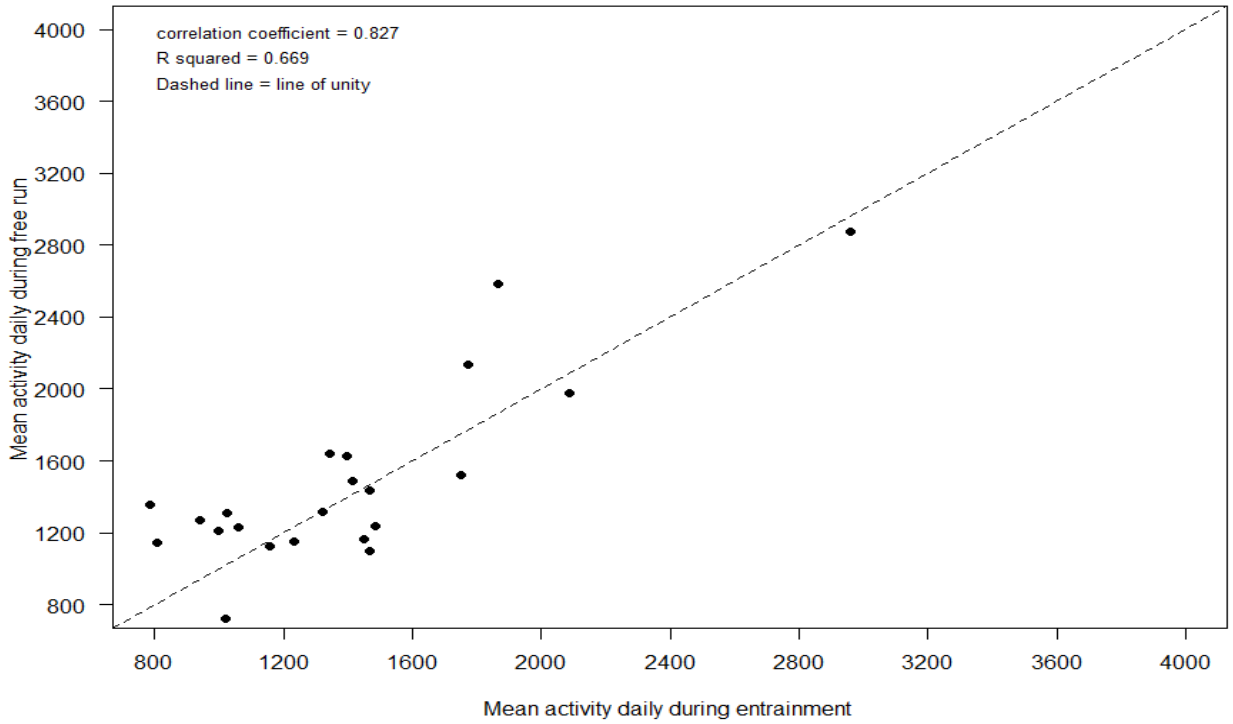


Bar charts showing the differences in daily mean activity for all fly groups between entrainment and free run and light and dark periods of entrainment respectively. *TilB* flies seem to be much more active than wild type flies in any situation, which may be the result of their lack of chordotonal organs.

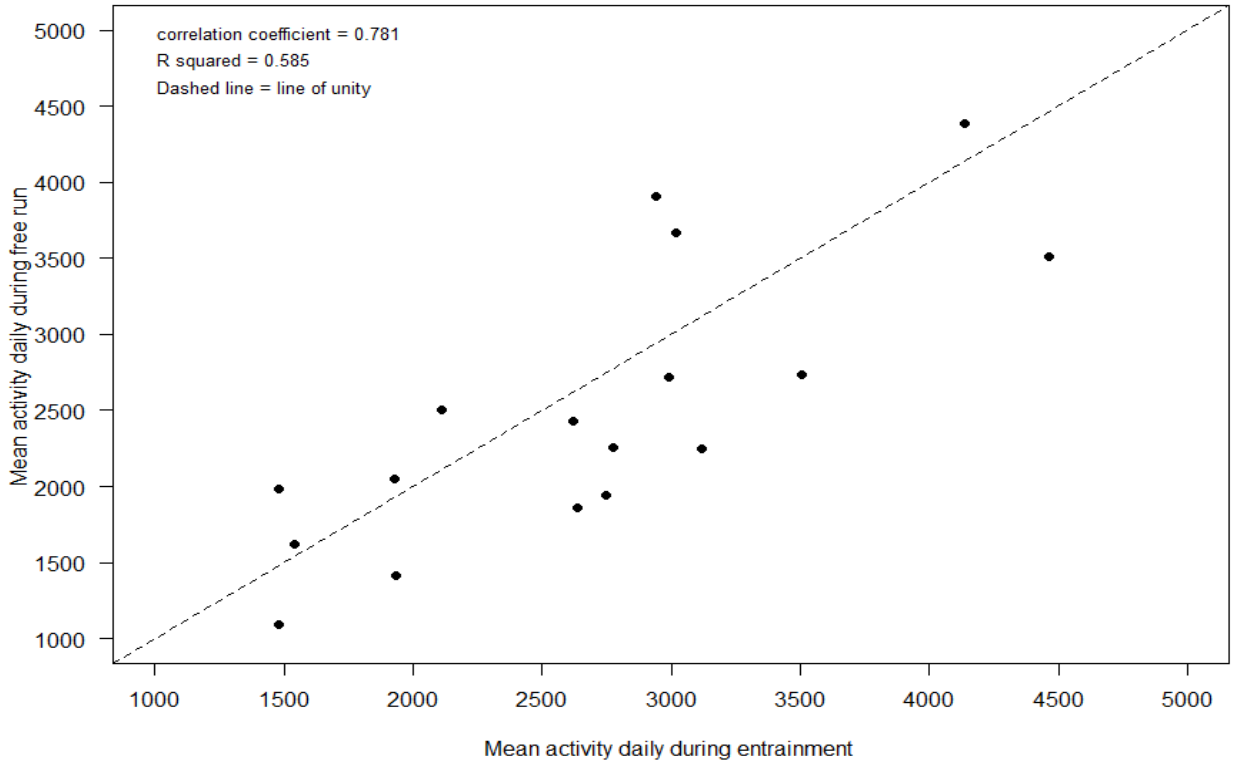
Appendix V: Hypothesis 1 tested for 3 days of entrainment only



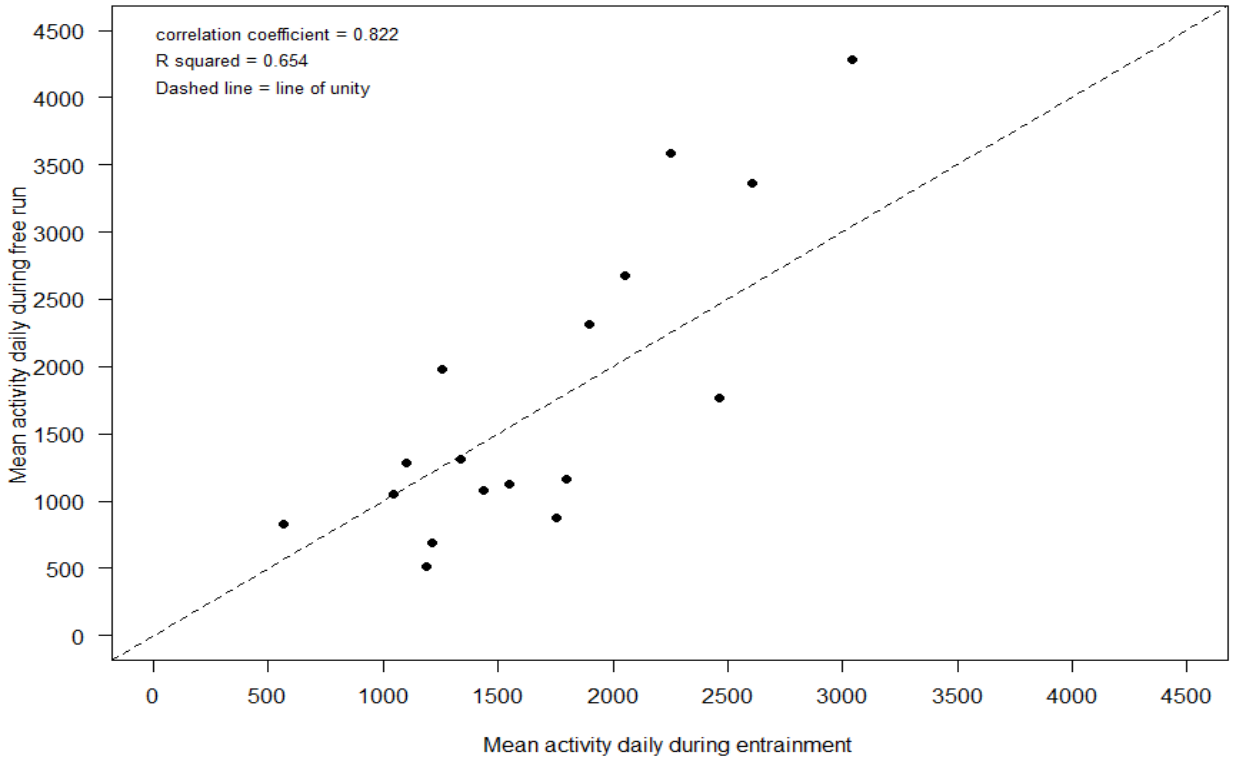
Mean daily activity during entrainment against free run for Canton S flies



Mean daily activity during entrainment against free run for Canton S flies that had been exposed to pymetrozine

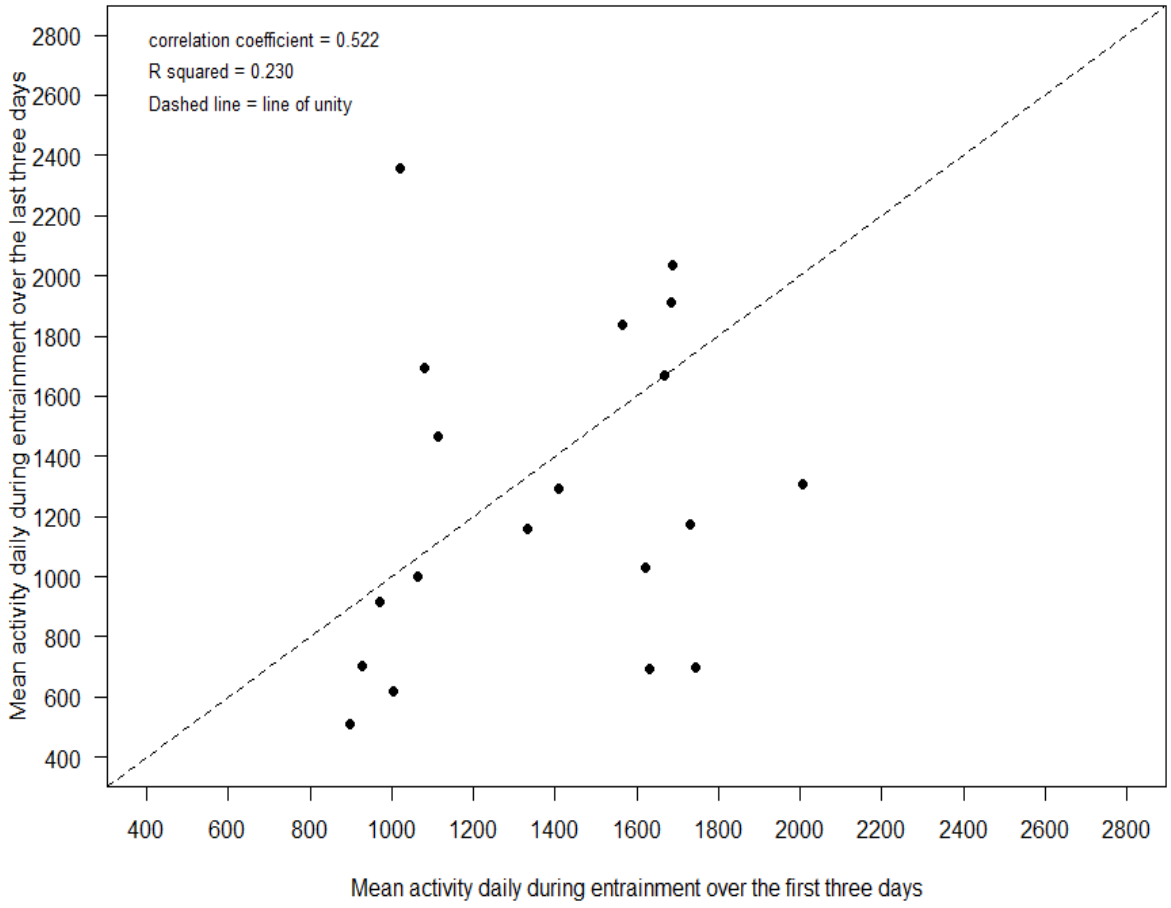


Mean daily activity during entrainment against free run for tilB flies

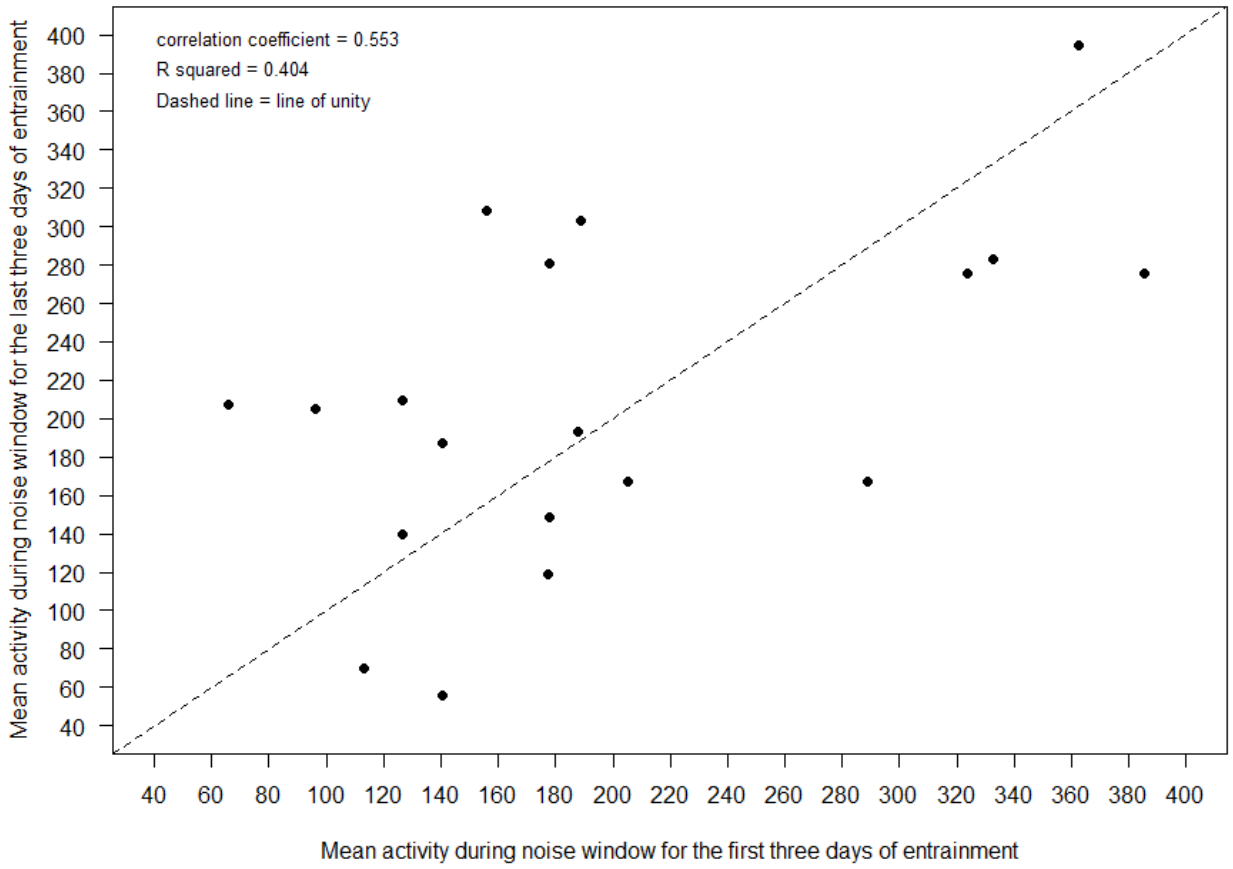


Mean daily activity during entrainment against free run for tilB flies that had been exposed to pymetrozine

Appendix VI: Investigating relationships during Canton S entrainment



The first investigation looked at the relationship between daily mean activity in the first three days of entrainment as compared to activity in the last three days of entrainment for Canton S flies. As can be seen from the above figure, there is basically no correlation between the two with a correlation coefficient of 0.522 being found. This could be the result of the data lost in the latter part of the third day of entrainment however and so a correlation cannot be entirely discounted.



After this, the relationship between mean activity in the noise window over the same time periods was looked at. Once again, there is essentially no correlation between the two, and in this case the data lost was relatively minimal. Thus it seems that mean activity in the first few days of entrainment is not a good predictor of mean activity at the end of entrainment.