DIFFERENTIAL CIRCADIAN BEHAVIORS IN AQUATIC ANNELIDS

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ABSTRACT OF THE THESIS

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In aquatic annelids, locomotion has proven to be a perplexing phenomenon because of the intricacies with which it is regulated. These animals are capable of either swimming or crawling (or both) depending on species. Studies of *Erpobdella punctata*, a temperate aquatic leech, have shown that each of these locomotion methods are differentially regulated according to an innate circadian rhythm. Swimming occurrences are regulated by an internal oscillator resulting in predictable circadian patterns while crawling is not, resulting in time-independent crawling outputs. The difference in these output pathways seems implausible, but could possibly be caused by each method of locomotion having independent output pathways from a circadian oscillator.

In this study, an automated motion capture experiment was designed to quantitatively evaluate various species of aquatic annelids for circadian-regulated crawling rhythms. *Erpobdella punctata* was used as a positive control for circadian behavior, but the experimental design used was unable to differentiate between swimming and crawling. To isolate crawling behaviors, a phylogenetic approach was

utilized by analyzing species closely related to *E. punctata* for comparison. *Helobdella robusta* and *Mesenchytraeus solifugus* - both of which are obligate crawling annelids - were tested for innate rhythms to determine whether lack of crawling regulation is found in other species as well. Simultaneously, attempts were made to isolate circadian oscillatory genes from each of the three species in question via the Polymerase Chain Reaction. Comparisons were made of known *Drosophila melanogaster* circadian regulatory genes clock, cycle, doubletime and cryptochrome with the *Capitella teleta* genome in an attempt to find annelid circadian sequences.

Amplified annelid sequences successfully showed no sequence similarity to any known genes in NCBI archives, nor in the *Capitella teleta* genome. However, *H. robusta* and *E. punctata* showed measurable crawling rhythm with close to a 24 hour period (while *M. solifugus* did not), implying future probing would be required to rule out the existence of a molecular oscillator in these species. The activity study also implies that lack of crawling rhythm in *E. punctata* is either unique to the species, or that previous studies regarding this behavior are flawed.

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INTRODUCTION

Circadian rhythms, internally regulated biorhythms synchronized to a ~24 hour period (Yerushalmi and Green, 2009), have proven to be a fundamental element of life. The existence of these rhythms is well documented among various animal species, and have been identified in other eukaryotic phyla as well (e.g. plants; Yakir et al. 2011), emphasizing their putative importance for life on Earth. These rhythms manifest themselves in many ways throughout the many branches of life in which they occur, but the most obvious manifestation is in overt behavioral change.

Current theories suggest rhythms developed to help organisms anticipate environmental changes that could adversely affect their survival (Yerushalmi and Green, 2009), such as the transition from day to night. Organisms that developed these rhythms would theoretically be better fit for survival in their particular environments, possibly by capitalizing on these daily changes to avoid predators, or by knowing what times of day their prey is most active. The exception to this rule seems to be organisms in extreme environments that lack daily cycles such as reindeer (Stokkan et al. 2007), which experience 24 hour days of dark or light depending on the time of year. However, in areas with regular, 24 hour cycling environments, animals with circadian rhythms appear to flourish.

A study of an aquatic annelid species *Erpobdella punctata* (Angstadt et al. 1997) showed that these organisms are no different in that they exhibit daily cycles of behavior. One peculiar finding of this study, however, was that these worms only had identifiable circadian rhythms while swimming, but not while crawling. The study was

built around a subjective evaluation of the prevalence of swimming and crawling behaviors, wherein each 5 minute period was given a binary value of 1 or 0 depending on whether or not the behavior in question occurred. This time series was then evaluated statistically to look for the existence of rhythms.

The results of this study imply that these worms could have two different regulatory pathways of their movements, but the experimental design is critically flawed. Ultimately not all activities are equal, and a more objective evaluation of activity would be required to truly understand the nuances of these rhythms. To achieve this, a video-recording setup was established to assess the circadian rhythms of *E. punctata* using a frame-by-frame pixel difference calculation. This setup gives a quantitative output that directly correlates to activity levels, because higher levels of activity generate a higher frame-to-frame pixel difference, therefore giving a higher output level. This completely removes human subjectivity from the equation, but therefore makes the differentiation of behavior types impossible.

With a new experimental design in mind, a phylogenetic approach was adopted to gain insight into *E. punctata*'s possible lack of crawling rhythm. Two species of annelids were tested alongside *E. punctata*: *Helobdella robusta*, a temperate climate, aquatic leech found in local ponds; as well as *Mesenchytraeus solifugus*, colloquially known as ice worms, aquatic annelids found in glacial ice/water mixtures on the Alaskan coast (Shain, et al. 2001). Though their locomotion methods vary slightly, neither of these worms is capable of swimming, and the decision to test them was made based on

this fact. While *E. punctata* has a documented swimming rhythm, lack of crawling rhythm in these species could indicate an inherited lack of crawling rhythm from a common ancestor.

Coupled with this, a biomolecular approach was utilized to identify the agents of a molecular oscillator in the species in question. Four known circadian regulatory genes were selected, including clock, doubletime, cycle, and cryptochrome, and alignments were made of their *Drosophila melanogaster* and *Mus musculus* orthologs with the *Capitella teleta* genome. Protein alignments were used for the generation of degenerative primers, which were then used for PCR probing to assess whether these genes were present in the annelid genome.

Worms were recorded for 7 days using a motion detection program, and their rhythms were then evaluated. *E. punctata* was confirmed to have a robust circadian rhythm of approximately 24.25 hours in length, and *H. robusta* was found to have a strong rhythm as well, with an approximate period length of 20.75 hours. The PCR probing was unable to find any definitive evidence of circadian oscillators within these species, which will need to be further explored given the results of their behavioral studies. *M. solifugus* was found to have a weak, sporadic rhythm of approximately 23.25 hours, which corroborates a lack of evidence found for a circadian oscillator via PCR.

MATERIALS AND METHODS

Specimens were either collected manually or purchased from commercial suppliers (thanks to Shirley Lang for making the trip to Alaska to collect the ice worms used in this experiment). Worms were maintained in culture in Dr. Shain's lab by Shirley Lang and Ralph Saunders until the time of the experiment. The temperate climate worms (*E. punctata* and *H. robusta*) were each kept in communal bowls at 18 ˚C until the time of experiment, where they were fed black worms *ad libitum*. They were immersed in filtered water supplemented with 300mg/L of Instant Ocean aquarium salt. *Mesenchytraeus solifugus* were stored in a cold room at 4 ˚C in communal containers filled with glacial melt water.

Each experiment lasted 7 days and tested four individuals. The first four days were LD (light / dark) 12:12 entrainment and the last three were DD (constant darkness). The "light" portion of the experiment was illuminated by lights with a peak wavelength at ~480 nm, while the "dark" portion was illuminated by lights with wavelengths at or above 700 nm. Studies have shown that polychaete (ancestral annelid) eyes have high sensitivity to wavelengths of light between 400 and 520nm (Yingst et al., 1997), and are almost completely insensitive to wavelengths above 540nm. Temperatures were held constant throughout the duration of each experiment; 18˚ C for *E. punctata* and *H. robusta*, 4˚C for *M. solifugus*. Subjects were not fed for the duration of the experiment.

Erpobdella punctata subjects were housed in individual sections of a custom-built tank that allowed water flow-through between sections to a reserve tank. This allowed for water changing throughout the week if it proved necessary without directly interacting with the worms, or breaking the visual plane of the camera. Worms were kept in a Percival Intelius chamber, and constantly filmed by a computer running the motion detection program. The tank was filled with 10 liters of fresh water.

Helobdella robusta subjects were housed in individual sterile Petri dishes with 10 mL of fresh water and stored in a light-proof container within a temperature controlled refrigeration room for the duration of the experiment. For *M. solifugus*, the same experimental setup was used as with *H. robusta*, with the exceptions that these worms were placed in Petri dishes of glacial water instead of filtered water.

Motion Detector Program

The camera was connected to a computer running an open source motion detection program simply called “Motion Detector,” modified specifically for this purpose by John Wang. The program assessed the images generated by the camera in real time, creating a frame-by-frame pixel difference reported as an average percentage of total pixel change over a given output time. Higher output values correspond to higher levels of movement during a given capture period. The output interval used was 5 seconds. The camera used was a Logitech C270 HD camera, modified to film in the infrared spectrum.

Data Manipulation

Raw output data was first normalized to the maximum movement levels for each worm to ensure each made an equal contribution to the total movement data. Normalized movements were then summed, and 5 second outputs were summed into 15 minute increments, giving a total of 96 data points per species per day. Data was then detrended for baseline and amplitude using a circadian rhythm analysis Microsoft Excel add-in called BRASS. This series was then analyzed using an add-in of Image J called Actogram J. With Actogram J the series were evaluated for period via Fourier Transformation and Lomb-Scargle periodograms, and an average amplitude behavioral chart was created, with which period fit was assessed using a cosinor least-squares regression analysis. The equation used for this evaluation is $f(x)=a*\cos((2\pi/P)*t+\phi)+M$, where a = amplitude, P = calculated period, t = time, ϕ = phase angle, M = mesor of wave. A minimized value of $(f(x)-y)^2$, where y is the average activity at any given time point, was calculated to find the best fit cosine function. All calculations were carried out in Image J and Microsoft Excel.

PCR Probe and Sequencing

Clustal W alignments were made of a series of circadian rhythm gene products from *D. melanogaster* coupled with their vertebrate orthologs. Alignments were made between doubletime and CKI-epsilon, cycle and bmal 1, and the clock and cryptochrome proteins from each respective species. These alignments were made against the *Capitella teleta* genome, from which degenerative primers were made. The primers used were:

Clock; Approximate product length of 201 bp

Forward: NGARTGGAARTTYYTNTT

Reverse: CCADATCCAYTGYTGNCC

CKI-ε/Dbt; Approximate product length of 234 bp

Forward: NMGNGTNGGNAAYAARTA

Reverse: YTCCATNACCATNAYRTT

Bmal1 / Cycle; Approximate product length of 702 bp

Forward: NGANGGNTTYYTNTTCGT

Reverse: NTKRTGRWARWAYTCRTA

Cryptochrome; Approximate product length of 285 bp

Forward: NGGNGGNGARACNSARGC

Reverse: RTARAARWAYTCNCKCCA

Primers were used to probe *E. punctata* and *H. stagnalis* genomic DNA, as well as an *M. solifugus* cDNA library, via Polymerase Chain Reaction. PCR products were run through a gel with electrophoresis, and bands of approximate desired length were cut and purified utilizing a Promega SV PCR cleanup kit. The purified product was then cloned using an Agilent Technologies Strataclone PCR cloning kit, from which colonies were chosen for sequencing. A Promega SV miniprep kit was utilized to isolate the DNA, which was then sent to GeneWiz for sequencing. Results were analyzed with the program Chromas and were viewed against the NCBI and *C. teleta* genome databases for a sequence match via BLAST search.

RESULTS

PCR Amplification and Sequencing

In *H. stagnalis* and *M. solifugus*, bands of an approximate desired length were isolated for both Dbt and Cryptochrome primer sets, but cloning and sequencing provided no identifiable results. All sequences proved to be empty vectors or sequences that returned nothing in either database tested, indicating the amplified sequences likely weren't part of the circadian rhythm genes we set out to isolate. *Erpobdella punctata* genomic data never produced bands of desired length through PCR amplification.

Activity assessment

All data presented is based on total activity levels. Because of this, since *E. punctata*'s locomotion is comprised of both swimming and crawling behaviors, the identified rhythm is associated with both movement types. *H. robusta* and *M. solifugus* activity is limited to crawling, so any defined rhythms can strictly be attributed to these crawling occurrences.

Figure 1 summarizes the phylogenetic relationships between the three worms in question. *M. solifugus* is the outgroup, delineating from *H. robusta* and *E. punctata* below the class level. Phylogenetic data for relationship derivation was retrieved from the Integrated Taxonomic Information on-line database, http://www.itis.gov.

Each species demonstrated a measurable diurnal rhythm – a daily behavioral rhythm corresponding to an external modulator's cycle – which is illustrated in Figure 2. In each case, subjects either ceased activity or showed a significant decline during "day," and had increased activity during "night." *Erpobella punctata* and *H. robusta* (Figures 2A, 2B) show the stereotypical response, with daytime activity levels declining towards zero by the fourth day of entrainment and nighttime activity levels remaining very high. *Mesenchytraeus solifugus* (Figure 2C) showed extremely high constitutive movement levels contributing to a smaller dichotomy between day and night activity levels, but the diurnal rhythm displayed is still clearly visible.

The first evidence of an endogenous rhythm is visible in these time series as well, seen during the final three days of the *E. punctata* and *H. robusta* experiments. Approximately 12 hours following the final offset of light, each species undergoes a trough in activity levels. This trough comes during the first portion of subjective light. Troughs continue periodically from that point forward, providing the first evidence of endogenous rhythms in these organisms. This also shows evidence of a dampening rhythm, as the activity levels in each species decline gradually as the experiments progress. *Mesenchytraeus solifugus* shows no such evidence of a rhythm, with no trough during the first portion of subjective light, and activity levels transitioning through a diminishing series of plateaus throughout the final three days.

Detrending the raw data (Figure 3) corrects for the dampening patterns and makes rhythm assessment easier, particularly for *E. punctata* who demonstrated the

most exaggerated dampening effects. The final three days of these series were tested for period via Fourier analysis (Figure 4), resulting in period estimates of 24.25 hours for *E. punctata*, 25.75 hours for *H. robusta*, and 28.5 hours for *M. solifugus*. Each of these period estimates is reasonably close to 24 hours, constituting one of the requirements for an endogenous circadian rhythm. A test for the fit of the estimated periods (Figure 5) shows a strong fit of the 24.25 hour period *E. punctata*, indicated by good behavioral grouping between subjective day and night. *Helobdella robusta* and *M. solifugus* didn't show the same grouping for their respective period estimates, each demonstrating erratic peaks scattered throughout the circadian day, and activity peaks during the subjective day – the opposite of trends displayed during the diurnal rhythm established during entrainment. Under a properly entrained endogenous rhythm, the representative cosine wave of free-running rhythms should closely resemble that of the entrainment phase calculations (Figure 6). Lomb-Scargle analyses (Figure 7) for period resulted in estimates of 20.25 hours and 23.25 hours for *H. robusta* and *M. solifugus* respectively. This resulted in much better behavioral grouping and a closer resemblance to the entrainment representative wave for *H. robusta* (Figure 8A), indicating the Lomb-Scargle estimate for period was far better than that found through Fourier analysis. Despite this added period estimate however, *M. solifugus* still shows poorly grouped behavioral trends compared to the other two species (Figure 8B), with peak activity levels occurring at 12.13 circadian hours, the border time between subjective day and night. This indicates that *M. solifugus* didn't entrain as well as the other two species,

making this identified rhythm weaker than those identified in *E. punctata* and *H. robusta*.

DISCUSSION

The phylogenetic relationships between *E. punctata*, *H. robusta* and *M. solifugus* are summarized in Figure 1. *Mesenchytraeus solifugus*, as the outgroup, was included as a potential point of comparison for the other two species. This study confirms previous *M. solifugus* studies illustrating their light avoidance behaviors (Shain et al. 2001), which is clearly visualized in Figure 2. Despite extremely high constitutive activity levels, *M. solifugus* showed activity peaks during the night time hours, illustrating a distinct diurnal rhythm. This trend disappears upon the end of the entrainment cycle however, with activity levels remaining elevated rather than troughing, making *M. solifugus* stand out from the other two worms. This created a state of continued activity elevation, clearly diverging from the diurnal rhythm illustrated throughout entrainment. Both *E. punctata* and *H. robustaí* experienced a trough in activity during the first portion of "subjective light," or the time starting 12 hours after the final light phase of entrainment. This trough is indicative of an internal modulator of activity gauging the change from day to night. In both *E. punctata* and *H. robusta*, presumably in anticipation of day's onset, the worm's activity decreased, despite the fact that the light never turned on.

Establishing a free-running period is expected of an endogenous circadian rhythm, but if entrainment is successful, the animals are expected to display similar behavioral trends to what they develop during the entrainment phase. For this reason *M. solifugus* is different when compared with the other two species. Immediately following the cessation of the light phase, *M. solifugus* maintained elevated behavioral

levels for approximately 30 hours rather than troughing like *E. punctata* and *H. robusta*, showing a massive departure from behavioral trends established during entrainment. Both of the tests for period used (Fourier Transform and Lomb-Scargle analysis) are susceptible to the identification of harmonics of rhythms, and despite the significance levels of the periods found, it is possible that longer periods of activity were simply ignored because of the detrending treatment to the data. Identification of this rhythm lends itself to the possibility of a much longer period rhythm in *M. solifugus* which would better represent its movement patterns, possibly in the 36-48 hour range. Ultimately the tests identified a 23.25h period for *M. solifugus*, but a closer look at the data shows that there were exaggerated over-arching activity trends that may be indicative of a much longer period of behavior. *Erpobdella punctata*'s data contained an example of this phenomenon in Figure 4A, which shows a second maximum period at ~16 hours. The data has been properly detrended for periods within this time-frame, and the 24.25 hour period represents the data well, but the 16 hour rhythm is still identified as a possible period for *E. punctata*. This is likely a harmonic of the 24.25 hour rhythm.

More telling than the differences between *M. solifugus* and the others were the similarities between *E. punctata* and *H. robusta*, which was the primary point of comparison for this study. Initial findings suggested that *E. punctata* lacked circadian crawling behavior, which created a dichotomy between the two types of locomotion available to the species. Assuming the previous findings were correct, crawling, a behavior which *E. punctata* shares with some of its closest relatives, would be

unregulated by a circadian oscillator, while swimming alone would show circadian regulation. This study was unable to directly address this issue, but it seems to have at least shed some light on why this behavioral abnormality was been found to be true, while simultaneously taking the research one step further.

Each worm had a stereotypical response to light associated with nocturnal animals: during the “day” their activity declined, and during the “night” their activity increased (Figure 2). This time-dependent variation of behavior carried over to the the constant darkness portion of the experiment for each worm, making identification of a rhythm simple and subjectively easy to spot after the data manipulation (Figure 3). Another common characteristic to these worms was the decreasing activity output over time – commonly referred to as rhythm dampening – visualized in the raw time series. This dampening implies that the primary factor maintaining activity amplitude could be the light-dark transition, meaning these worms could naturally exhibit successively weakening activity with difficult to identify rhythms in constant dark conditions. The dampening of activity level in *E. punctata* is more exaggerated than in *H. robusta*, but this is caused by *E. punctata*’s maximum output level being much higher than *H. robusta*’s because of *E. punctata*’s ability to swim. Dampening is corrected by detrending with respect to amplitude and thus is not accounted for in the period estimates, but the decline in movement could contribute to the lack of behaviors to observe, thus reinforcing the original postulate that no crawling rhythm exists in *E. punctata*.

Note: While no definitive rubric for differentiating between swimming and crawling has been established in this study, Figure 2 contains total activity data for *E. punctata*, and during the final three days of recording the maximum behavioral peak was only ~30% of the total maximum movement value, to which the data points were normalized. The decreasing behavioral amplitudes could be the result of fatigue or hunger as the experiment proceeded, but the fact that amplitudes decreased means it is possible these last three days show mostly crawling behaviors. For this analysis, this behavior was attributed to swimming rhythmicity for comparison, but a better defined correlation between output level and activity type could be all that is required to address this issue (see Other Thoughts).

One major pitfall of Angstadt et al. (1997) is that no movement period was identified for statistical analysis, and the LD entrainment schedule was simply extended for the remainder of the experiment to identify subjective light and day. In this study, each of these worms exhibited periods that were slightly different from 24 hours, 24.25 hours in *E. punctata* and 20.25 in *H. robusta*, which could explain the lack of identification of a rhythm. Unless subjects display an endogenous rhythm of exactly 24 hours to coincide with an established diurnal rhythm, activity trends will slowly drift from a given time of day to a time regulated by an internal oscillator. Simple coincidence could account for the fact that a swimming rhythm was identified and a crawling rhythm was not.

The setup is further confounded by the fact that despite using a visual recording setup, researchers introduced a stone behind which the worms were given the opportunity to hide. This was done as a courtesy to the animals to give them a place to stay out of the light, but every moment the worms are behind a rock is one moment that their movement is not being recorded. Working with *E. punctata* in culture revealed how frequently they would seek shelter within their environment, and providing them with a place to hide could only skew the results from their intended course. For this reason, no shelter was provided to the worms in this study so that all moments of activity could be captured and analyzed. Identification of *H. robusta*'s rhythm justifies this element of experimental design, and the fact that a period was identified implies *E. punctata* would also likely have circadian crawling regulation.

Future Directions

Previous studies indicate a difference in daily regulation of *E. punctata*'s variable locomotion types, but what causes this phenomenon is never addressed. Extreme environments that lack daily cycles tend to give rise to animals lacking overt circadian rhythms in lieu of rhythms more favorable to their environment, such as the reindeer's variable melatonin secretion patterns (Stokkan et al. 2007), or sandpiper arhythmicity during breeding season in Alaska (Steiger et al. 2013). *Helobdella robusta* and *E. punctata* both live in local lakes and are subject to similar environmental stresses, with the only exception being that *E. punctata* is capable of swimming while *H. robusta* can only crawl. Finding such a weak rhythm in *M. solifugus* was not entirely unexpected

because of the widely varying daily cycles of their native environment in Alaska, but the activity assessment of *H. robusta* shed some light on the possibility for crawling rhythms in *E. punctata*. Extending this test to other species of annelids, possibly worms that are capable of swimming but instead prefer to crawl, would make behavioral isolation simpler, and make a more direct comparison with *E. punctata* possible.

Secondly, there is currently no known molecular circuit for circadian rhythms in annelids, while one exists for mammals (Albrecht and Eichele, 2003) and insects (Ogawa et al. 2008). Attempts were made to isolate genes of known circadian regulatory function from *E. punctata*, *M. solifugus*, and a close relative of *H. robusta*, *Helobdella stagnalis*, but failed. Eventually the search was abandoned. Establishing an annelid molecular model would be a logical next step to this experiment. This model could provide a molecular explanation for the presence of rhythm in one species and the lack of rhythm in another, as well as providing more insight into whether *E. punctata* has two different regulatory pathways for their varying locomotion methods or not.

Other Thoughts

This study sought to compare the circadian behaviors of these three species of annelids, however there were several underlying motivations which bear mentioning. First, we sought to successfully automate the analysis of overt annelid behaviors via this experimental setup, which was achieved successfully. The design of the motion detection program allowed for an objective, quantitative analysis of activity without a prerequisite for measurable displacement – between which an important distinction

must be made. Similar research on other species have often relied on behavior being translated into mechanical output for measurement, such as the reliance on running wheels in mouse studies. This study has successfully proven that no such setup would be required in future research, and more accurate representations of overt behavior can be made with simpler and cheaper experimental protocols.

The pixel-difference calculation creates an environment in which movement does not need to be correlated with displacement for measurement, and can simply be measured as movement. This makes cross-species comparisons possible despite different locomotion methods. Lacking the anterior / posterior locomotion method shared by *E. punctata* and *H. robusta*, *M. solifugus* proved incapable of any measurable displacement across the plastic Petri dish surface, and in each case wound up in the same spot on day 6 that they were placed on day 0. *Helobdella robusta* and *E. punctata* movement patterns are ideal for moving across smooth surfaces. Coupled with *E. punctata*'s ability to swim, each worm was capable of much higher mobility than *M. solifugus*, yet *M. solifugus* still had higher constitutive movement values.

Secondly, this study was designed to reassess findings by Angstadt et al., (1997), hence the decision to include *E. punctata* as a positive control for circadian behavior. Their methods of evaluation relied on binary assignments of movement types per 5-minute interval, and thus had low resolution. An automated setup would vastly improve over this experimental design. Identification of a rhythm in *E. punctata* therefore proved as a point of comparison as well as a proof of concept for the new experimental design,

with one major caveat. Since the process was entirely automated and hardware limitations forced an output time of 5 seconds, I was left with no ability to differentiate between swimming and crawling behaviors like they were able to do in the original study. The rhythms identified in *E. punctata* are movement-type neutral, and have no indication whether they were derived from swimming or crawling outside of output amplitude. The ribbon-like swimming patterns of the leeches gave rise to much larger outputs than their crawling behaviors, leading to the assumption that any swimming rhythm would be able to be identified, even if none existed in their crawling. The identified rhythms are attributed to their swimming behaviors for comparison, but that doesn't rule out the existence of crawling rhythms, since that element of the previous study wasn't able to be evaluated.

The precision with which these data points are generated lends itself to more comprehensive applications, and there is much more potential to be drawn from this experimental design. With smaller output windows, side-by-side analyses of video recordings and motion detection could be used to establish movement thresholds on a second-by-second basis, as I initially intended to do with this study. These defined parameters could then be used to differentiate between swimming and crawling (or for terrestrial species, between walking and running) allowing for a far better understanding of the ways in which organisms behave throughout the day. Setups like these simply need more exposure, but once wide-spread within the scientific community, their impact on behavioral studies will prove useful.

Ultimately this study addresses the circadian behaviors of *E. punctata*, *H. robusta* and *M. solifugus*, and in doing so it addresses general issues with the study of annelids. Annelids are robust organisms with a wide array of environments. The inclusion of *M. solifugus* in this study illustrates that annelids aren't merely found in gardens and lakes, but can exist in environments as extreme as glaciers. Few organisms display such widely varied distributions, which makes annelids a powerful resource to the biological community. Phylogenetic studies of annelids, such as this one, can rely upon multitudes of outgroups against which comparisons of phenomena can be made. Annelids are also generally easy to raise and care for in a laboratory environment, making them ideal organisms for many studies that would otherwise turn to more popular models, such as *D. melanogasters* or *C. elegans*.

Despite their ease of use in scientific pursuits, historically worms have been a group of organisms overlooked by the scientific community. The first taxonomy of animals developed by Linnaeus originally grouped all invertebrates together as *Vermes*, or "worms," and wasn't changed until Lamarck decided to further classify invertebrates years later (Clifford, 2004), officially establishing the annelid taxon. According to Angstadt et al. (1997), the identification of *E. punctata*'s endogenous rhythm was the first ever identified for an aquatic annelid. Prior to that study, researchers noted that *E. punctata* and other leeches were considered to be arrhythmic, with irregular activity patterns not correlating with the time of day. The reasons for this belief were anecdotal however, and were likely propagated by lack of understanding and flawed research of the animals in question.

The Angstadt et al. (1997) study was flawed in this way as well. By not creating an experimental design precise enough to account for all elements of activity, researchers overlooked a fundamental element of *E. punctata* behavior, one which this study elaborated on by drawing comparisons between *E. punctata* and *H. robusta*. The inclusion of *M. solifugus* took this comparison further, showing annelids behave in much the same way as other organisms, demonstrating rhythms in natively rhythmic environments (*E. punctata* and *H. robusta*), and having no rhythm in arrhythmic environments (*M. solifugus*). With the prevalence of circadian rhythms being identified today, and studies identifying earthworm rhythms as early as 1974 (Burns et al. 2009), the assumption that aquatic annelids obey different laws than the majority of species on Earth is a dubious one, which this study was designed to dispute.

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FIGURES

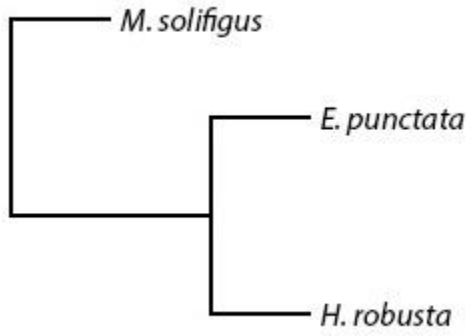

Figure 1: Species relationship dendrogram, *M. solifugus*, *E. punctata*, and *H. robusta*. *Mesenchytraeus solifugus* is the outgroup, distinguishing itself from *E. punctata* and *H. robusta* at the subclass boundary, belonging to *Oligochaeta* as opposed to *Hirudinea*. Classification data retrieved 7/25/2013 from the Integrated Taxonomic Information System (IT IS) online database, http://www.itis.gov

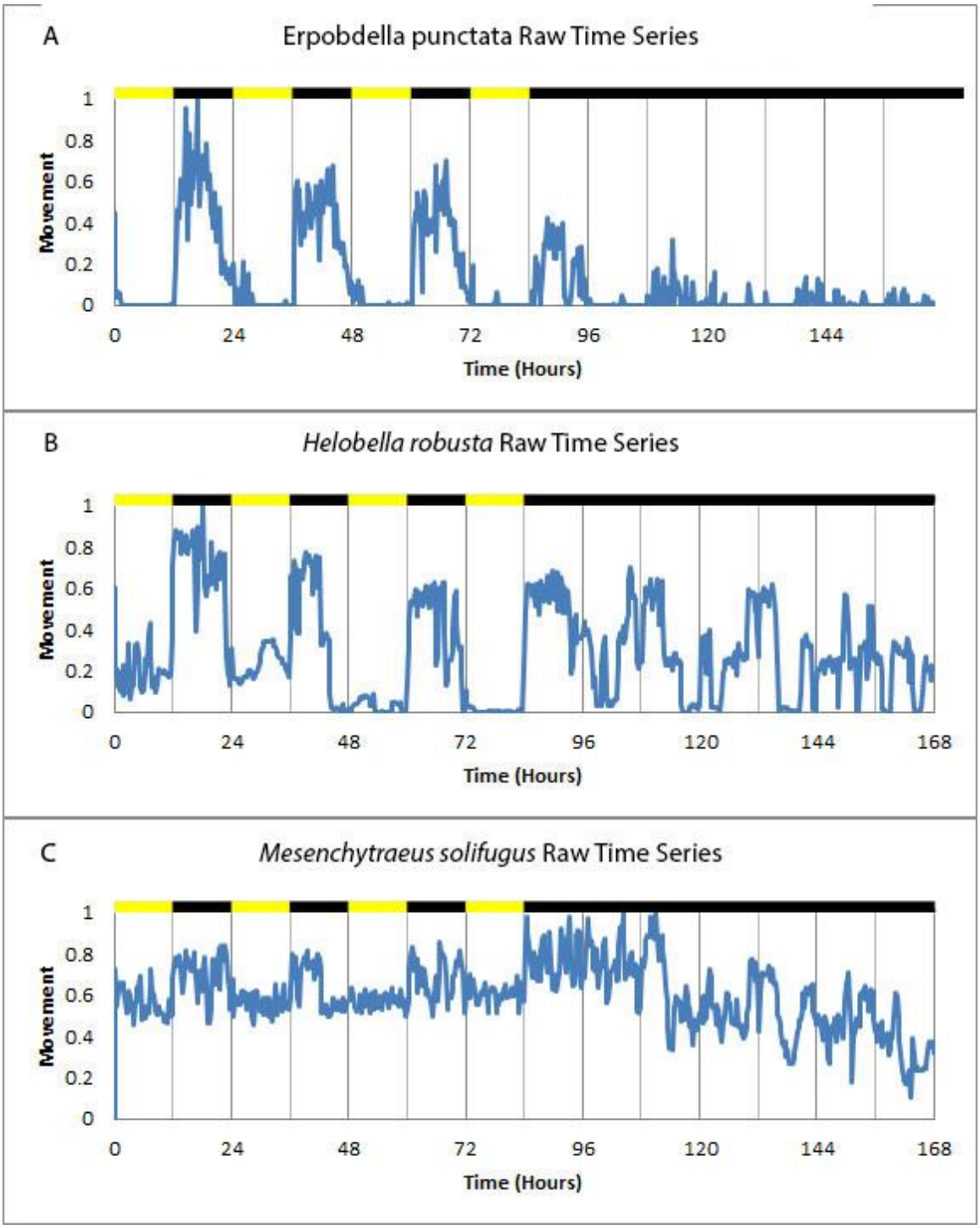

Figure 2: Diurnal rhythms in raw time series. Five second intervals summed into 15 minute increments. Light bar indicates light at the given time in the experiment; Yellow = L, Black = D A) Raw *E. punctata* time series. B) Raw *H. robusta* time series. C) Raw *M. solifugus* time series.

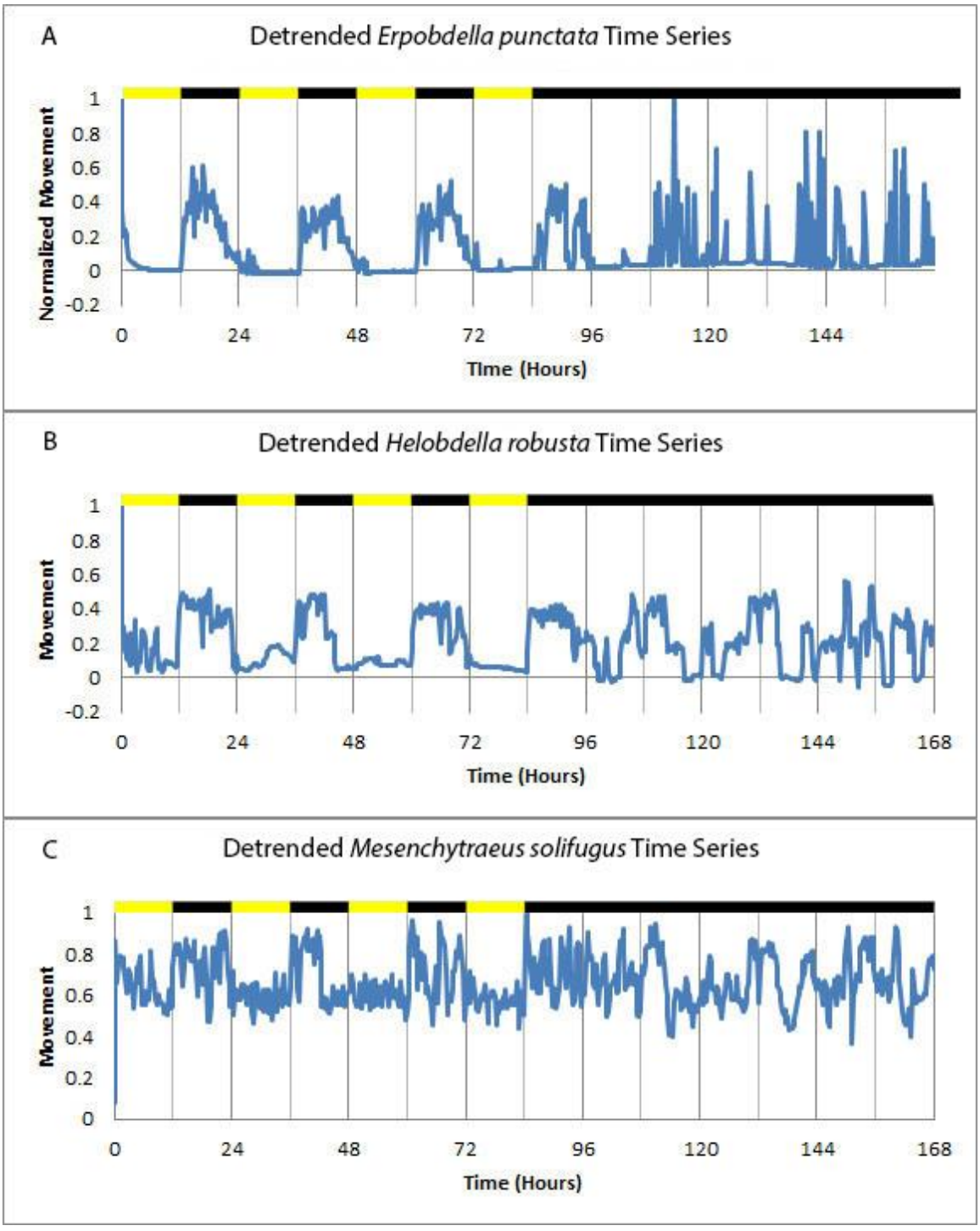

Figure 3: Endogenous rhythm dampening reduction. Constant dark activity values (hour 96+) utilized for period estimates of free-running rhythm. A) Detrended *E. punctata* time series. B) Detrended *H. robusta* time series. C) Detrended *M. solifugus* time series.

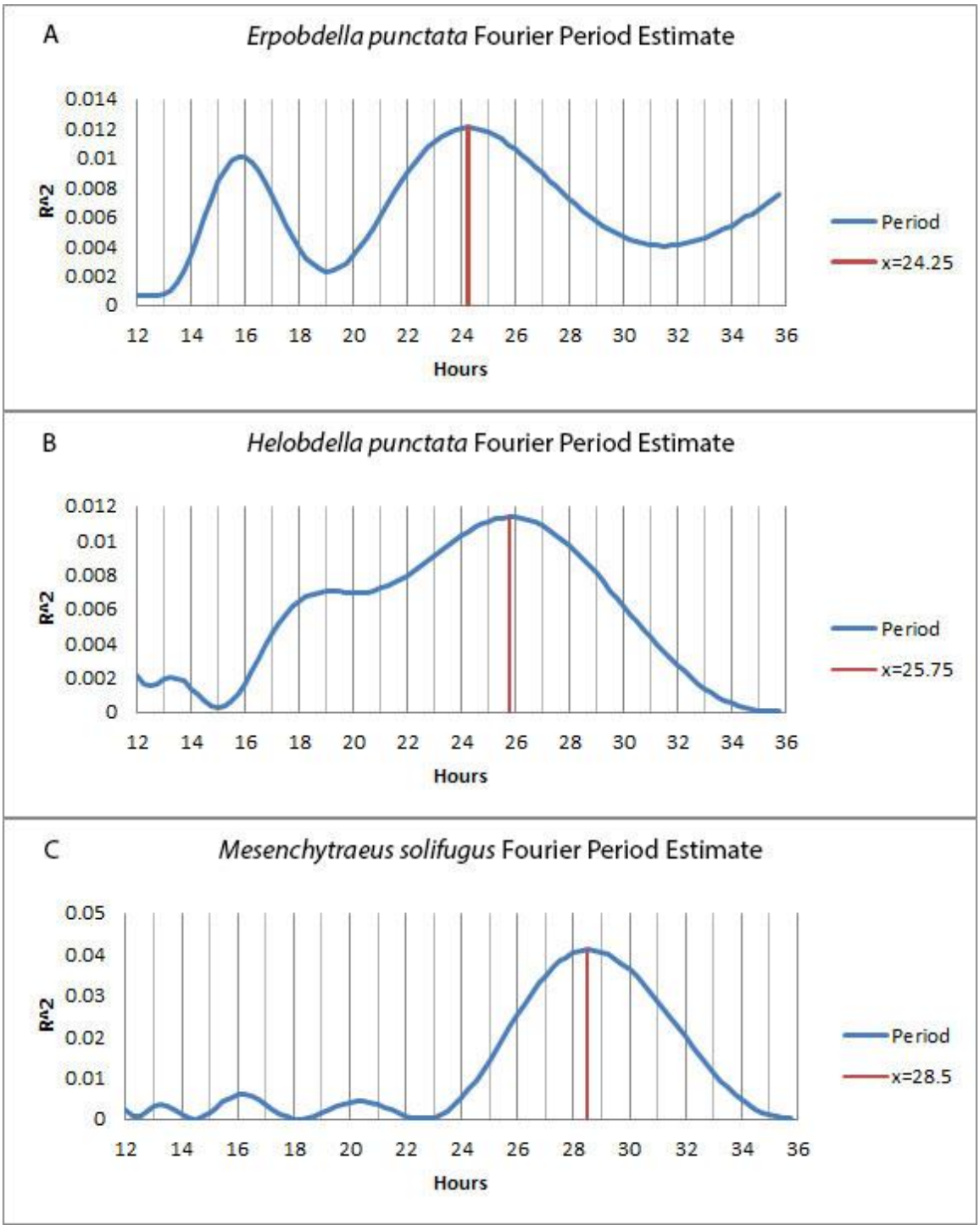

Figure 4: Fourier Period Estimates. Y axis = frequency value, X axis = hourly period. A) *Erpobdella punctata* period estimate, primary peak at x = 24.25 hours. B) *Helobdella punctata* period estimate, primary peak at x = 25.75 hours. C) *Mesenchytraeus solifugus* period estimate, primary peak at x = 28.5 hours.

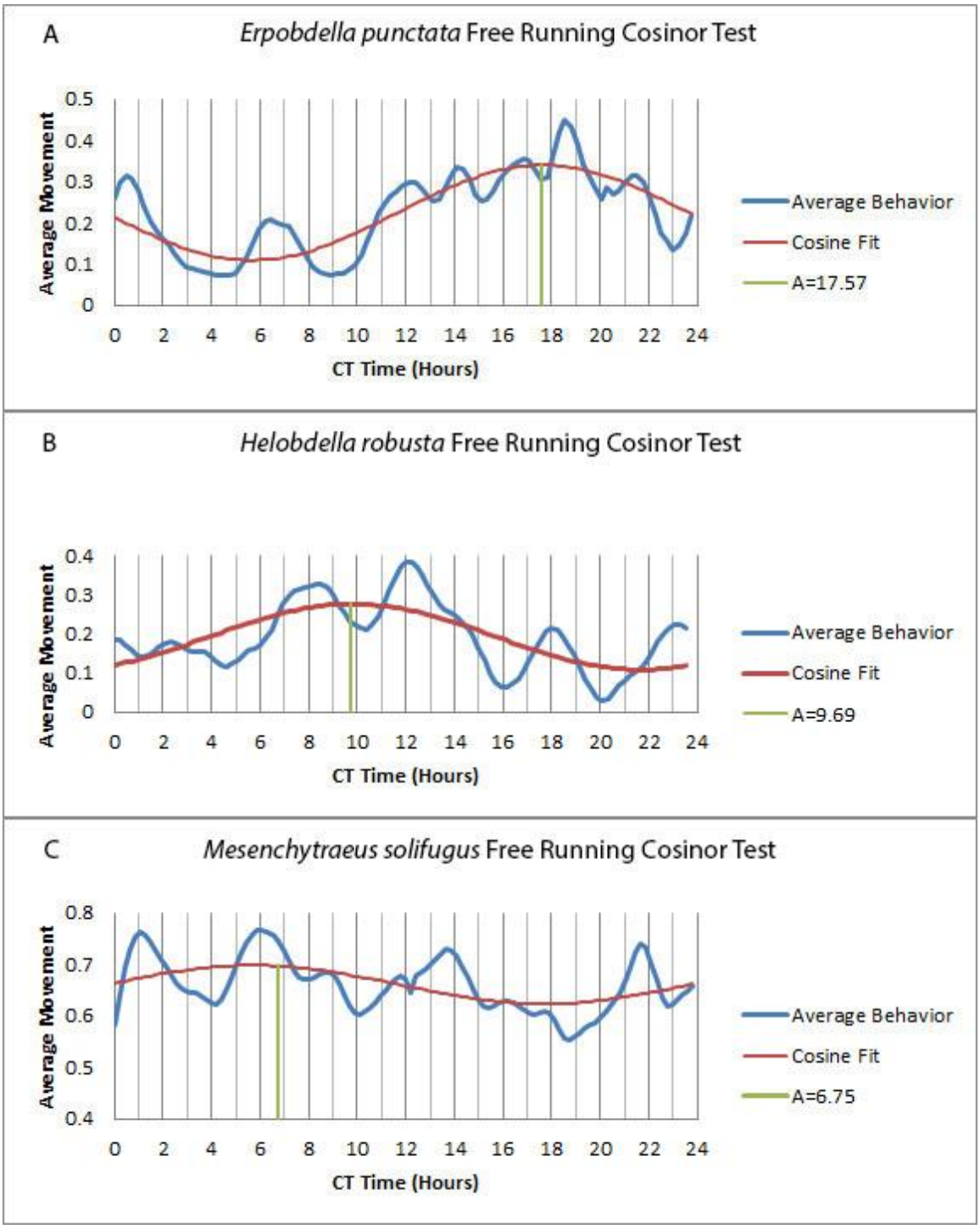

Figure 5: Period-fit tests for Fourier period estimates. Average data acquired for each time point across final three days of experiment. A) *Erpobdella punctata* fit test. Strong behavioral grouping indicates a strong fit of 24.25 hour period estimate. Acrophase A at 18.56 circadian hours, ϕ = -4.87 radians, amplitude a = 0.058. B) *Helobdella robusta* fit test. Multiple activity peaks indicate poor fit of 25.75 hour period estimate. Acrophase A at 9.69 circadian hours, ϕ = -2.59 radians, amplitude a = 0.084. C) *Mesenchytraeus solifugus* fit test. Multiple peaks indicate poor fit of 28.25 hour period estimate. Acrophase A at 6.75 circadian hours, ϕ = -1.49 radians, amplitude a = 0.037.

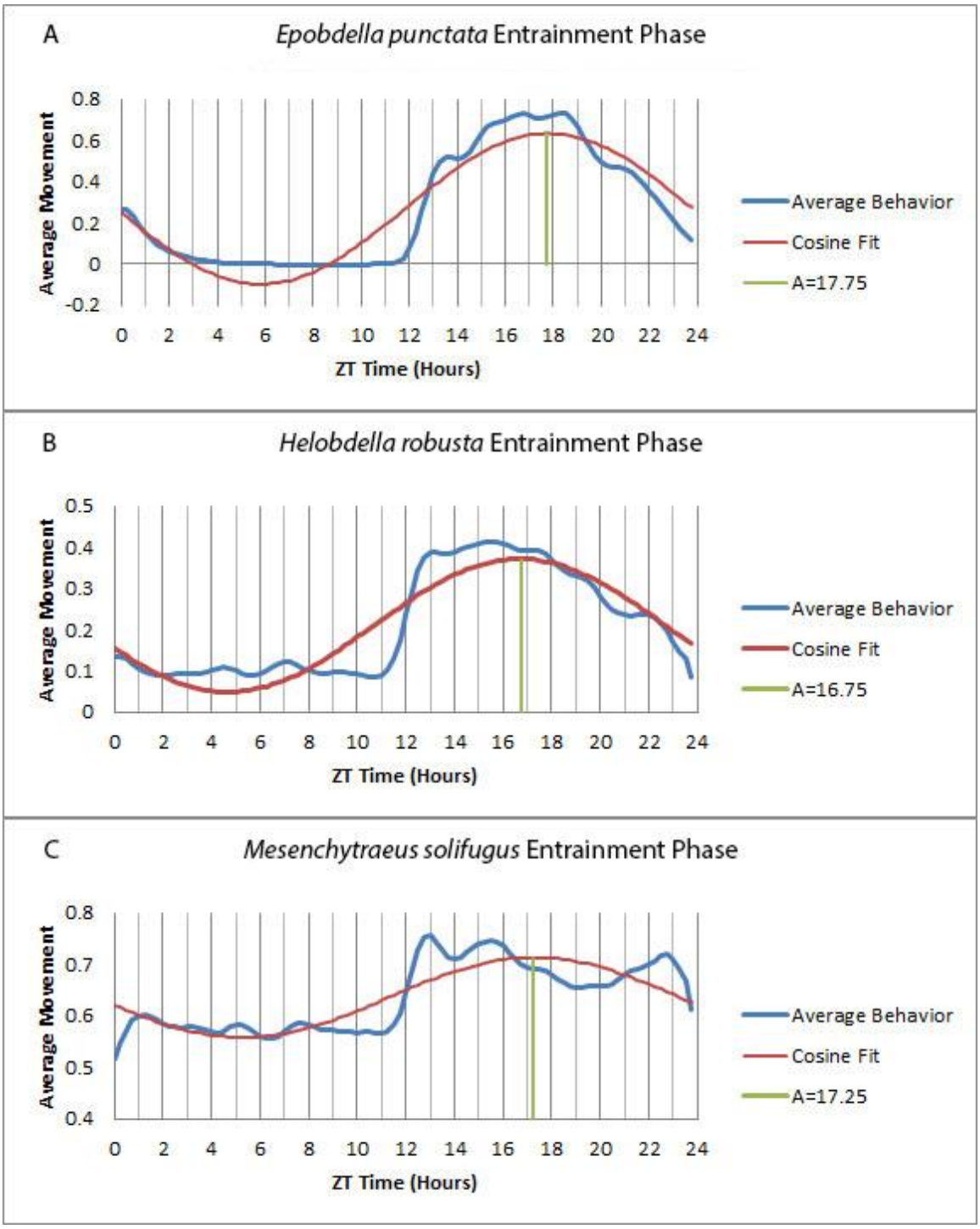

Figure 6: Cosinor entrainment phase estimates. Average data acquired for each time point across first four days of experiment. A) *Erpobdella punctata* entrainment phase. Acrophase A at 17.75 hours, ϕ = -4.66 radians, amplitude a = 0.18. B) *Helobdella robusta* entrainment phase. Acrophase A at 16.75 hours, ϕ = -4.37 radians, amplitude a = 0.16. C) *Mesenchytraeus solifugus* entrainment phase. Acrophase A at 17.25 hours, ϕ = -4.53 radians, amplitude a = 0.078.

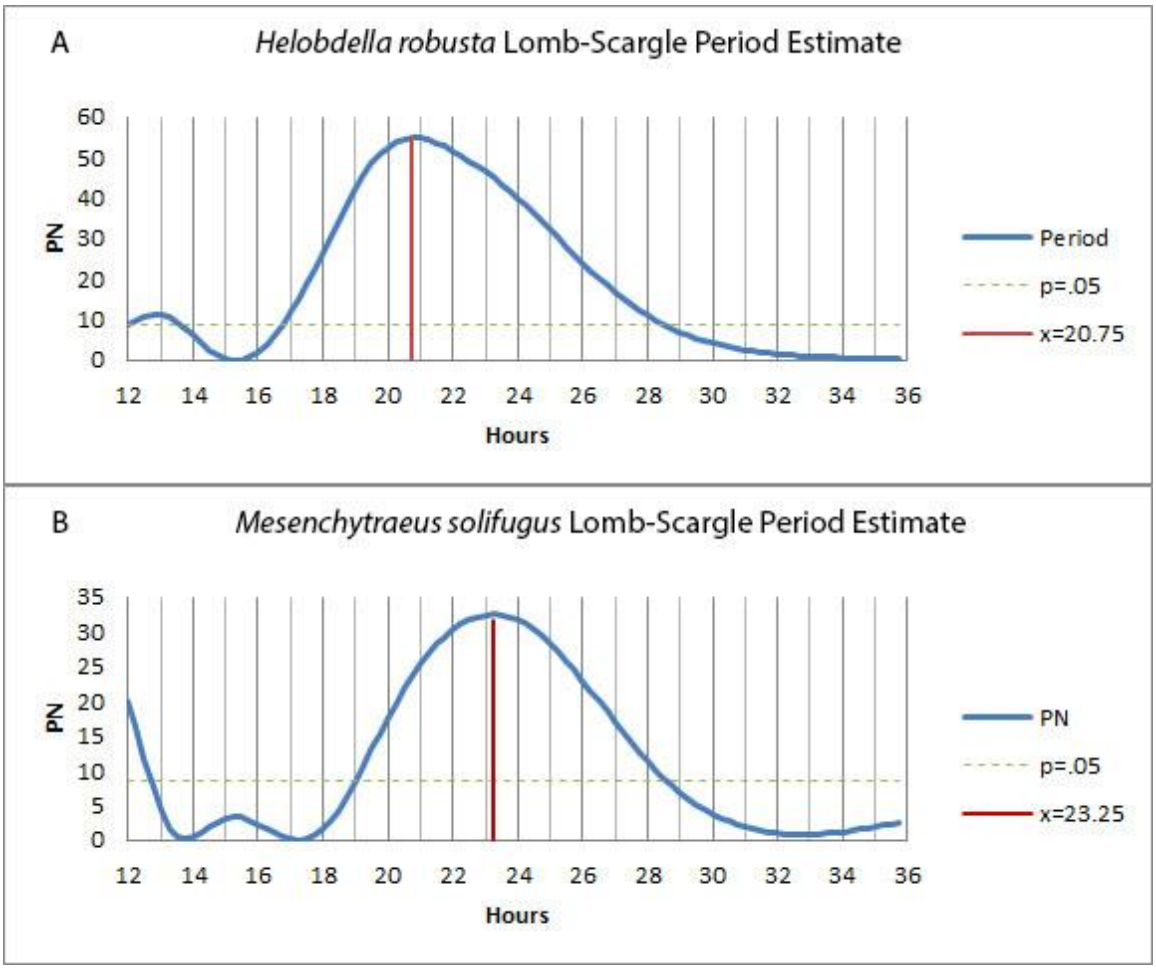

Figure 7: Lomb-Scargle period estimates for *H. robusta* and *M. solifugus*. Estimates made to replace Fourier estimates, which poorly represented rhythms in data. A) *Helobdella robusta* period estimate, primary peak at x = 20.75 hours. B) *Mesenchytraeus solifugus* period estimate, primary peak at x = 23.25 hours.

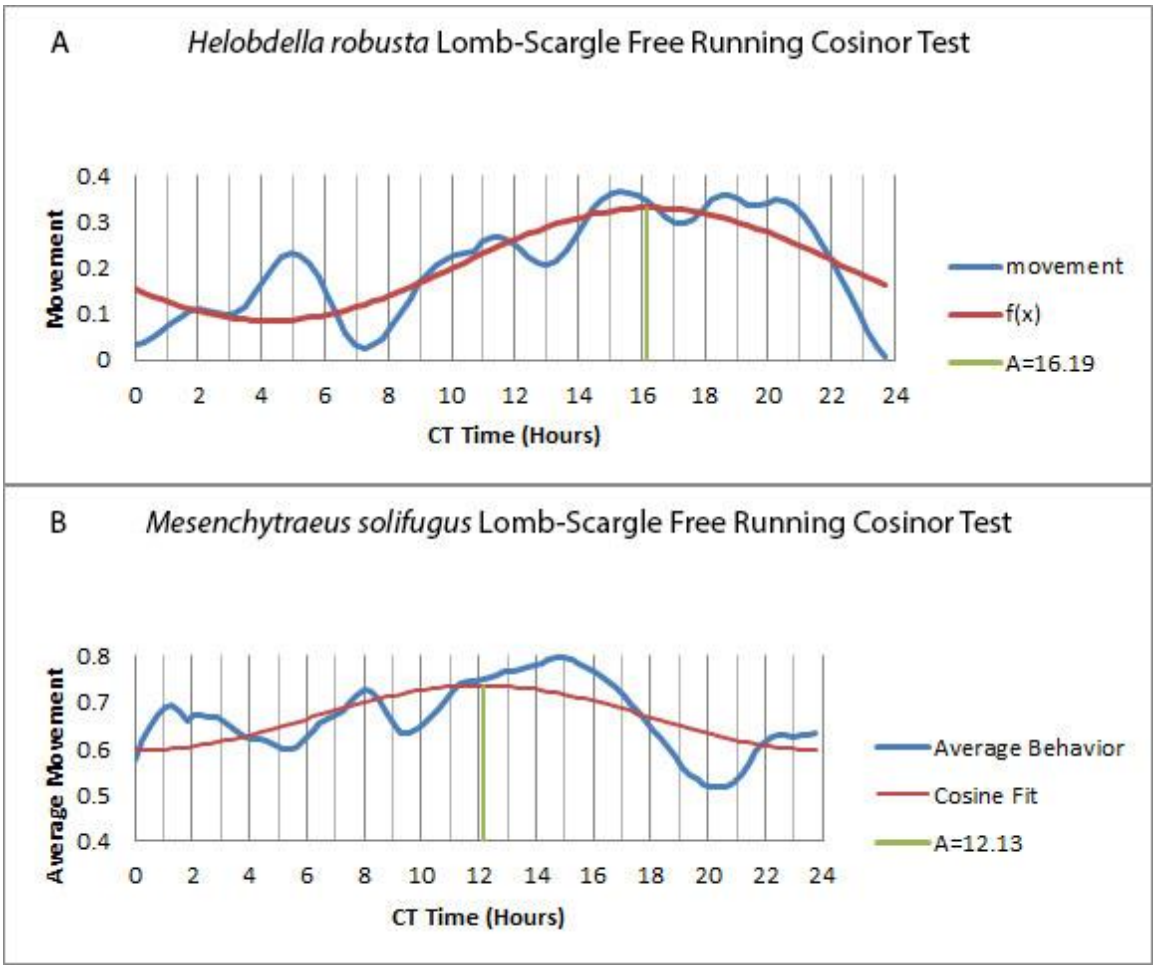

Figure 8: Period-fit tests for Lomb-Scargle period estimates. Average data acquired for each time point across final three days of experiment. A) *Helobdella robusta* fit test. Strong behavioral grouping indicate good fit of 20.75 hour period estimate. Acrophase A at 16.19 circadian hours, ϕ = -4.26 radians, amplitude a = 0.12. B) *Mesenchytraeus solifugus* fit test. Poor behavioral grouping and shift of acrophase relative to entrainment indicate poor fit of 23.25 period estimate. Acrophase A at 12.13 circadian hours, ϕ = -3.17 radians, amplitude a = .070.