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The Expression of Circadian Rhythms in the Non-Diapausing Mosquito Culex pipiens f. molestus

Natalie Rachel Epstein

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Abstract

Mosquitos within the *Culex pipiens* species complex are primary vectors of West Nile virus in North America. The most common member of this complex in New Jersey, *Cx. pipiens* f. *pipiens*, is adapted to a temperate climate and females enter a diapause state as adults each winter. In contrast, its co-occurring sister taxon, *Cx. pipiens* f. *molestus*, is unable to enter the diapause state and both males and females continue feeding and breeding during winter months. Females additionally continue to lay eggs. The *molestus* form can do so because it is highly adapted to urban environments and is predominantly found in manmade underground locations. Prior studies have shown that the genes associated with circadian rhythms (i.e. 'clock genes') also influence the photoperiodic induction of diapause in *Cx. pipiens*. Here I investigated whether New World *Cx. pipiens* f. *molestus* maintains circadian rhythms despite its inability to enter a diapause state. The adult emergence of mosquitos reared in 12:12 light:dark, constant light, and constant dark had circular means in or around early scotophase (dark period). The adult emergence of mosquitos reared in the contrasting cycle of 12:12 dark:light had a circular mean after lights on, which was scotophase in the incubator. These results indicate that circadian rhythms in New World *Cx. pipiens* f. *molestus* are entrained by environmental cues during the larval period. Genetic analysis using BLASTn compared the expression of known clock genes in *Cx. pipiens* f. *molestus* and the closely related species *Cx. quinquefasciatus*. All known clock genes were expressed in *Cx. pipiens* f. *molestus* and suggest that the lack of diapause in these mosquitos is unlikely to be caused by major inactivating mutations in known clock genes.

MONTCLAIR STATE UNIVERSITY

The expression of circadian rhythms in the non-diapausing mosquito *Culex pipiens* f.

molestus

By

Natalie Rachel Epstein

A Master's Thesis Submitted to the Faculty of Montclair State University

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

May 2020

College of Science and Mathematics

Department of Biology

Thesis Committee:

_____
Dr. Matthew Aardema, Thesis sponsor

_____
Dr. Lisa Hazard, Committee member

_____
Dr. Kirsten Monsen, Committee member

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THE EXPRESSION OF CIRCADIAN RHYTHMS IN THE NON-DIAPAUSING MOSQUITO

CULEX PIPIENS f. MOLESTUS

A THESIS

Submitted in partial fulfillment of the requirements

For the degree of Masters of Science

By

NATALIE RACHEL EPSTEIN

Montclair State University

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Introduction

The body temperature of an ectothermic organism is determined by the temperature of its environment. In temperate climates, seasonal changes typically bring corresponding changes in the temperature of the environment. Ectothermic organisms have evolved biological adaptations in order to cope with the colder winter months. There are two common strategies used by ectothermic organisms to deal with colder temperatures. These are known as the freeze-tolerance strategy and the freeze-avoidance strategy. An ectotherm that uses the freeze-tolerance strategy can freeze a portion of its body water to control the amount of ice formed within the body. Freeze-avoidance ectotherms produce antifreeze proteins within their bodies to prevent the formation of ice within the body (Voituron et al. 2002). These two strategies allow ectothermic organisms to live in harsher climates.

Insects are ectothermic organisms that are sensitive to seasonal changes due to their smaller size (Lazzari and Insausti 2008). While some insects have adapted the freeze-tolerance and freeze-avoidance strategies to survive the colder months, others will initiate diapause to survive the winter (Bale and Hayward 2009). Diapause is a state of dormancy in which growth and development of the insect is halted until environmental conditions are once again favorable (Tauber and Tauber 1976). To prepare for diapause, an insect must accumulate fat to ensure that it has enough energy to survive. It then needs to find a suitable shelter that reduces exposure to cold temperatures and dangerous environmental conditions. Finally, the insect must undergo other physical changes that are required for survival (Meuti et al. 2015). Diapause is common in insects living in temperate regions where the summers are warm with long days and the winters are cold with short days (Denlinger 2002; Lazzari and Insausti 2008).

One of the common initiators of diapause is the change in the environment's photoperiod, or amount of daylight. This is also known as the photophase. (Saunders 2002). For temperate insects this information is important because it indicates when the seasons are changing (Adkisson 1966). Insects use the shortening of the photophase and the increase of the scotophase, or dark period, as an exogenous cue indicating the need to enter diapause. For diapause to be initiated an insect must store the information about the environment's photoperiod and translate it into hormonal cues that are needed to initiate any biological processes (Meuti and Denlinger 2013, Saunders 2002). For certain adult insects, diapause is signaled by a delayed release in juvenile hormones (JH) instead of environmental changes. In the mosquito *Culex pipiens*, females that undergo diapause do not release JH after adult eclosion. Instead they experience no growth in their ovarian follicles and will not seek out a blood meal (Denlinger and Armbruster 2014). Once the corpora allata starts to synthesize JH diapause is terminated, and the ovarian follicles start to grow (Denlinger 2002). Nondiapausing females of this species will experience the release of JH by the corpora allata sometime after the adult emerges and will immediately seek out a blood meal (Denlinger and Armbruster 2014).

Prior studies (Meuti and Denlinger 2013, Ikeno et al. 2010) show that there is a connection between the use of photoperiod to initiate diapause and the genes that control circadian rhythms (i.e. 'clock genes'). Circadian rhythms are produced by an organism's internal clock in response to changes in the environment. There are four properties that the rhythms must adhere to, the first being that it must maintain endogeneity, or that the patterns will still run in the absence of environmental cues. The second property is that the rhythm reflects the environmental cycle, which is typically the 24-hour solar day. The third property is the maintenance of homeostasis across a variety of different variables, typically temperature. Finally, the rhythms

must be able to entrain to environmental variables, such as light (Andreani et al. 2015, Saunders 2002).

There are five primary clock genes associated with circadian rhythm that maintain the feedback loop. These genes are *timeless (tim)*, *period (per)*, *Clock (clk)*, *cycle (cyc)*, and *doubletime* (Saunders 2002). The basic model for circadian rhythm is the *Drosophila* molecular clock. *per* and *tim* are the negative elements in the loop and repress the expression of the positive elements *clk and cyc.* Overnight the proteins PER and TIM slowly accumulate outside of the nucleus in the cytoplasm and create a dimer. The morning light will then cause TIM to degrade, which will destabilize PER. However, they will start to accumulate again in the evening and move into the nucleus. There, PER-TIM will bind with CYC-CLK, which inhibits the transcriptional activities of the proteins. Once that happens DNA can bind at an E-box, and the loop is closed. The positive elements*, clk* and *cyc*, will then reset the cycle (Albrecht & Ripperger 2008, Andreani et al. 2015, Saunders 2002).

Circadian rhythms become ingrained in an organism's genome as the species evolves. The behaviors that are associated with the rhythms are not learned but are innate responses. However, external influences, such as temperature and light, can modify the behaviors. *Zeitgebers*, or environmental cues, can move the rhythm from endogenous to exogenous (Saunders 2002). A common environmental cue is the daily light-dark cycle an organism is exposed to (Lazzari and Insausti 2008). On a day-to-day basis, insects display a circadian rhythm that is approximately 24 hours with peak activity levels occurring at a particular time during that cycle. If an insect reared under 12:12 light:dark conditions is moved into a completely dark or a completely light environment it will still perform activities based on the cycle it was reared in (Meuti and Denlinger 2013). One example of this is the egg-hatching rhythm of the moth,

Pectinophora gossypiella. Moth eggs were raised at 20 $^{\circ}$C under either 24h light, 24h dark, or a 12:12 light:dark cycle The hatching of the eggs raised under 24h light and 24h dark conditions was aperiodic, which indicated there was not a certain period of the day in which the eggs would hatch. However, those raised in 12:12 light:dark displayed a rhythm where hatching happened shortly after dawn (Minis and Pittendrigh 1968). These experiments demonstrate the endogenous nature of an organism's circadian rhythm.

Studies have revealed that either mutating or using RNA interference (RNAi) to knockdown, or reduce the expression of, the clock genes will affect the photoperiodic induction of diapause (Meuti and Denlinger 2013). In a study performed on the bean bug, *Riptortus pedestris*, RNAi was used to suppress the expressions of *per* and *cyc* to determine the role these two genes play in the photoperiodic induction of diapause. When *cyc* expression was suppressed females bean bugs entered diapause when they were moved into a diapause-averting photoperiod. Under a diapause-averting photoperiod, the photophase is longer and female bean bugs typically experience ovarian development. On the other hand, the suppressed expression of *per* resulted in the aversion of diapause and ovarian development in diapause-inducing conditions. In a diapause-inducing photoperiod, the photophase is shorter and there is typically for no ovarian development occurring in female bean bugs. The bean bugs display a photoperiodic response that will be disrupted when the expression of *cyc* and *per* are suppressed. (Ikeno et al. 2010). This study demonstrates the role of clock genes in the induction of photoperiodic diapause.

Mosquito species in the *Cx. pipiens* complex are the primary vectors of West Nile virus in North America (Fonseca et al. 2004). In New Jersey the most common member of the complex is *Cx. pipiens* f. *pipiens*, or the Northern House Mosquito, which are adapted to

temperate, aboveground environments. Female *Cx. pipiens* f. *pipiens* are anautogentic meaning they require a blood meal before their first ovipostion (Byrne and Nichols 1999). Mating takes place in large open spaces, while flying when an adult female approaches a swarm of males (Byrne and Nichols 1999). Swarming activity and mating typically occur around twilight. After mating prior to winter, females will enter diapause and males will die off (Vinogradova 2000). These mated females then seek a blood meal and lay eggs the following spring, after they emerge from the diapause state.

Female *Cx. pipiens* f. *pipiens* require functioning clock genes to initiate diapause. Under long day, diapause-averting conditions, the supressed expression of *per, tim*, and cryptochrome2 (*cry2*) results in an elevated expression of pigment dispersing factor (PDF), which is known to play a part in circadian timing. The suppressed expression of these genes also leads to the non-diapause phenootype. However, if PDF expression is suppressed in the females that are reared in long day conditions, the diapause phenotype occurs. In short day, diapause-inducing conditions, the expression of *per, tim*, and *cry2* is elevated and the diapause phenotype occurs (Hand et al. 2016).

Cx. pipiens f. *pipiens'* co-occurring sister taxon, *Cx. pipiens* f. *molestus* is predominately found in urban environments and spends the winter months in warm, underground shelters. It has lost the ability to enter the diapause state (Byrne and Nichols 1997). It has been suggested that *Cx. pipiens* f. *molestus* populations may have arisen from local *Cx. pipiens* f. *pipiens* populations (Kothera et al. 2010). Since their sister taxon can diapause and diapause is considered an ancestral trait, it is possible that the loss of the ability to enter diapause is due to genetic differences.

The difference in the behaviors and physiology of the two mosquitos could be attributed to *Cx. pipiens* f. *molestus* adaptation to the underground environment (reviewed in Fonseca et al 2004). *Cx. pipiens* f. *molestus* are found in and adapted to manmade, underground environments with standing water (i.e. sewers, cellars, drainage pumps, subway tunnels, etc.) where light is either inconsistent or not available. In the warmer months *Cx. pipiens* f. *molestus* will emerge from their underground dwellings and feed on mammals (Bajwa and Zuzorsky 2016). Their aboveground sister taxon feeds on birds. While female *Cx. pipiens* f. *pipiens* diapause during the winter, male and female *Cx. pipiens* f. *molestus* continue to actively feed and breed. They are found to enter human inhabited areas and feed on humans. However, females do not require a blood meal for their first ovipostion (autogeny), but in order to keep breeding they must seek one out (Byrne and Nichols 1997).

It is thought that New World and Old World *Cx. pipiens* f. *molestus* have separate evolutionary origins (Aardema et al. 2020). Previously, a study done in Russia investigated the effect of light and temperature on the rhythm of adult eclosion in *Cx. pipiens*. Researchers found that under an artificial photoperiod and constant temperature Old World *Cx. pipiens* f. *molestus* have peak adult eclosion activity in the evening during early scotophase. This experiment looked at three different day lengths (12, 16, and 20h) and found the adult eclosion peak shifted to match the dark phase (Karpova 2005).

 In this study, I investigate circadian rhythms of New World *Cx. pipiens* f. *molestus* to determine if they maintained similar rhythms despite an inability to enter the diapause state. I postulate that New World *Cx. pipiens* f. *molestus* has lost its ability to express circadian rhythms. There are two observations to support this hypothesis. First, there is no consistent light cycle in the underground locations where *Cx. pipiens* f. *molestus* is typically found, and the photoperiodic

clock is important for the maintenance of circadian rhythm (Saunders 2002). Second, prior studies have found a link between clock genes and diapause (Meuti and Denlinger 2013, Ikeno et al. 2010). An inactivating mutation in known clock genes could simultaneously cause an inability to diapause and a loss of circadian rhythm. Adult emergence is one of the biological processes controlled by circadian rhythm in mosquitos (Vinogradova 2000). If New World *Cx. pipiens* f. *molestus* do not have a circadian rhythm then adult emergence will be arrhythmic no matter the light cycle during larval development.

Materials and Methods

Mosquito Maintenance. I kept adult *Cx. pipiens f. molestus* originating from New York City in cages where they were fed a diet of 8% sucrose solution. To collect eggs, I placed oviposition trays into the cages for 48 hours. After I removed the trays from the cage, egg rafts were collected, and each raft was placed into an individual jar with 50ml of dechlorinated water to ensure that each family was separate from one another. The larvae were fed a diet of TetraMin fish food, and individuals from the families were never mixed.

After hatching, I placed five to fifteen larvae from each family into one of four treatments: 12:12 light:dark (lights on at 06:00, lights off at 18:00), 12:12 dark:light (lights off at 06:00, lights on at 18:00), 24 hours of light, and 24 hours of dark. The jars containing the larva had 50ml of dechlorinated water. They were then moved to an incubator that was kept at 23 $^\circ$C and they were kept there until pupation.

Trials. After pupation, I transferred each pupa into individual Drosophila vials (Genesee Scientific) that contained approximately 25 mL of dechlorinated water and were labeled

according to family and light cycle. The vials containing the pupae were then moved into a dark chamber and placed onto one of four shelves. The dark chamber had a light regime of 12:12 light:dark (lights on at 06:00, lights off at 18:00). During the dark period, a red light would come on once every hour for one minute in order to help detect any emergence activity. It is important to note that after the fifth trial, I replaced the fluorescent light bulb with an equivalent LED bulb due to the fluorescent bulb burning out. The emergence of the adults was filmed using a YI 1080p home security camera.

Video technology has been used to film mosquito behavior and activity in laboratory settings previously. In a recent study the behavior of *Anopheles gambiae* and *Aedes aegypti* was filmed using a Flybox. The use of the device in this experiment demonstrated an easy way to monitor the behaviors for several days under light-dark and constant dark conditions (da Silva Araujo et al. 2020).

Video Analysis. I viewed all the video recordings and documented the times of all emergences that occurred during the trials. An emergence was identified as a mosquito emerging from its pupal case or a mosquito treading on the surface of the water (Fig. 1).
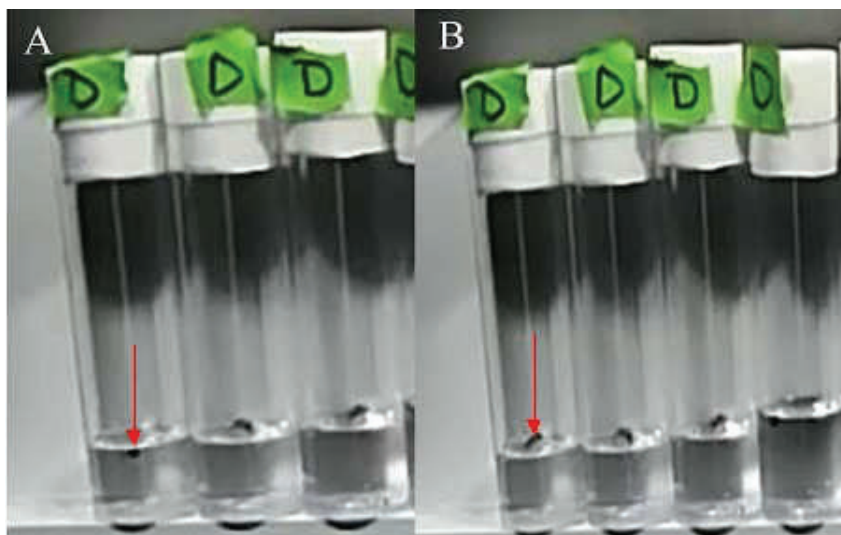
Figure 1. A) *Cx. pipiens* f. *molestus* pupae before emergence. B) *Cx. pipiens* f. *molestus* adult after emergence.

Statistical Analysis. I plotted the obtained emergence times on a clock (Lund et al. 2017) associated with the treatment using RStudio (RStudio Team 2015). In order to appropriately plot the times on the clock, I had to convert the minutes into decimals. For example, if an adult emerged at 12:30 and 30 minutes is half of an hour (i.e. 60 minutes), the time became 12.50. I analyzed the results using a Rayleigh Test (Lund et al. 2017) to test for uniformity with $\alpha = 0.05$. The aim for this test was to see if adult emergence occurred at a certain time of the day. I tested the null hypothesis that there is no pattern displayed in the adult eclosion of *Cx. pipiens* f. *molestus*.

Genetic Analysis. To assess potential genetic expression of known clock genes, the transcriptomes of two *Cx. pipiens* f. *molestus* samples, one from North America (Price & Fonseca 2015) and one from Europe (Honnen et al. 2006), were de novo assembled from data publicly available on NCBI-SRA using the program TRINITY v2.6.6 (Haas et al. 2013). I then used a nucleotide-to-nucleotide blast (BLASTn) to compare the nucleotide sequences of the two *Cx. pipiens* f. *molestus* samples to the already known and annotated nucleotide sequence of *Cx. quinquefasciatus* (Arensburger 2010).

Results

Emergences. The Rayleigh test found there was significant evidence of adult emergence occurring at a certain time of the day for all four treatments. For mosquitos reared under 12:12 L:D the test statistic was 0.6042 ($p < 0.001$) and for mosquitos reared under 12:12 D:L conditions it was 0.3413 ($p < 0.001$). The Rayleigh test statistic for constant light was 0.2051 ($p = 0.009$) and for constant dark it was 0.2301 ($p = 0.0045$).

I also found the circular mean to find the mean emergence time for the four treatments, which is indicated by an arrow on the clock. (Fig 2). The circular mean for 12:12 L:D was 21.96 (21:58) with a circular standard deviation of 2.26. For 12:12 D:L the circular mean was 10.14 (10:08) with a circular standard deviation of 1.91. Under constant light the circular mean was 20.25 (20:15) with a circular standard deviation of 2.22. Finally, the circular mean for constant dark was 17.84 (17:50) with a circular standard deviation of 2.42.
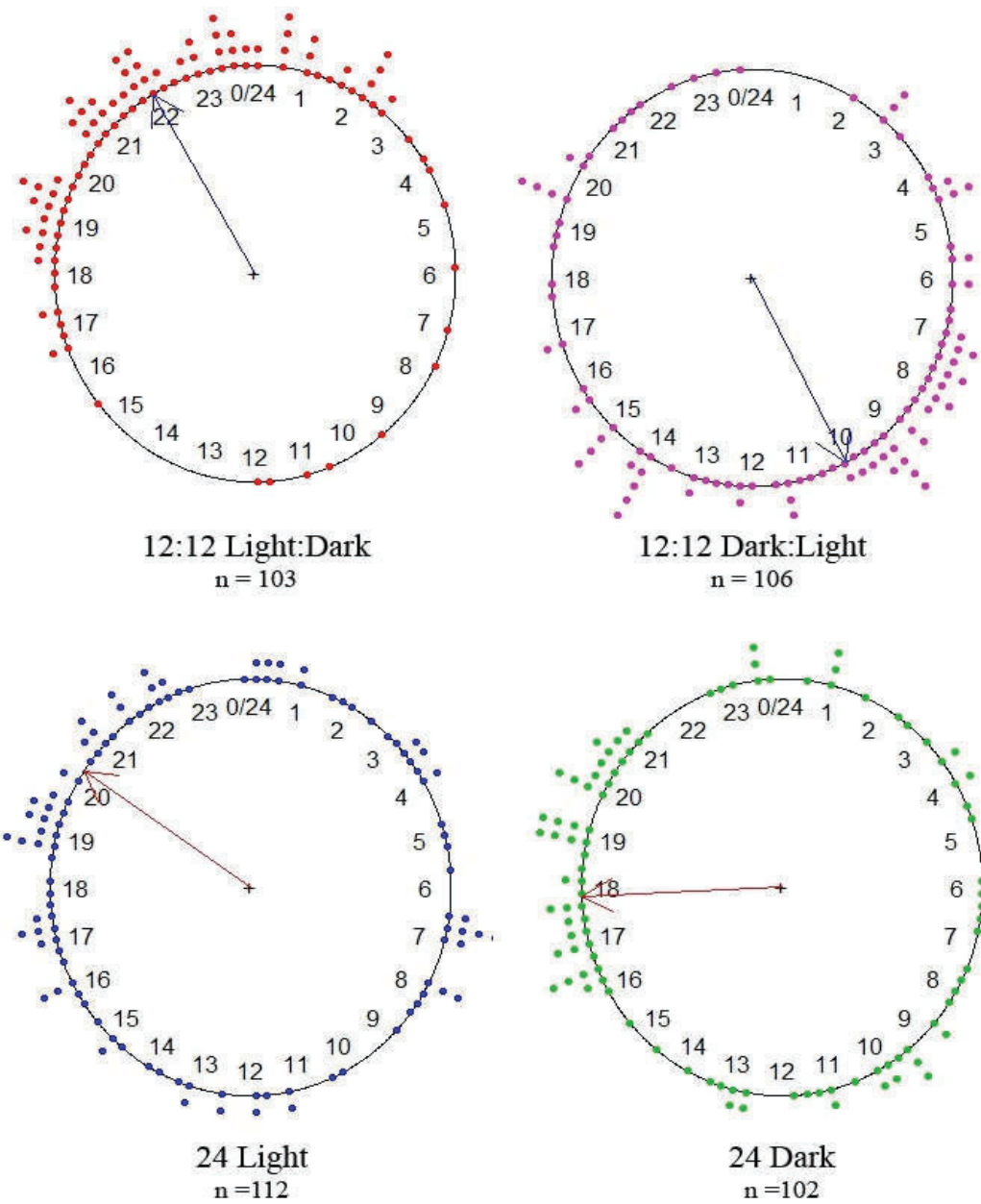
Figure 2. *Cx. pipiens* f. *molestus* adult emergences on a 24-hour clock, divided by treatment. Each dot represents one sample's emergence time and the arrows indicate the circular mean of the treatment.

Presence of Clock Genes. BLASTn hit percent similarity found that clock genes were present in both the North American *Cx. pipiens* f. *molestus* (M_USE_1) and the European *Cx. pipiens* f. *molestus* (M_EUR_1) (Table 1). The minimum percentage of nucleotide similarity between *Cx. pipiens* f. *molestus* and *Cx. quinquefasciatus* was set at 95%. Most genes in both *Cx. pipiens* f. *molestus* samples had over a 95% nucleotide similarity with the genes found in *Cx. quinquefasciatus*.

Table 1. Presence of expressed clock genes in North American *Cx. pipiens* f. *molestus* (M_USE_1) and European *Cx. pipiens* f. *molestus* (M_EUR_1). Accession numbers are from the reference *Cx. quinquefasciatus* genome. Given are the top BLASTn hits percent nulecotide similarities.

Gene Name	Accession Number	M_USE_1	M_EUR_1
cryptochrome-1	XM_001865552.1	97.358	96.443
cryptochrome-1	XM_001867860.1	95.222	95.499
cryptochrome 2	XM_001869421.1	95.536	95.474
clock protein	XM_001864988.1	94.612	95.449
timeout/timeless-2	XM_001842172.1	96.237	96.204
timeout/timeless-2	XM_001842173.1	97.124	97.224
timeless protein	XM_001848559.1	97.227	97.015
period protein	XM_001849247.1	96.624	96.275
circadian locomoter output cycles kaput protein, mRNA	XM_001843362.1	97.213	97.096
circadian locomoter output cycles kaput protein, mRNA	XM_001843363.1	94.915	95.206
lark	XM_001850644.1	96.747	99.145

Discussion

The results of this experiment show that New World *Cx. pipiens* f. *molestus* retains circadian rhythms despite its an inability to diapause. The Rayleigh tests for all four treatments showed significant evidence of adult eclosion occurring at near or during early scotophase. For mosquitos reared in the reverse photoperiod 12:12 D:L, scotophase occurred during 06:00 and 18:00. It also significant that the circular means for 12:12 L:D, constant light, and constant dark

are similar and occur during the time period state before. The genetic analysis found that the known clock genes are expressed in these mosquitos and it is unlikely that inactivating mutations in these clock genes cause the lack of diapause. These findings along with the qualitative data (Fig 2) contrast to my initial prediction that no rhythms would be displayed.

Although I predicted that no rhythms would be displayed, the results I obtained are not surprising. The results for New World *Cx. pipiens* f. *molestus* reared in 12:12 L:D matched the results found in Karpova's study of Old World *Cx. pipiens* f. *molestus* in that a majority of adult eclosion would take place in early scotophase. Karpova's study also found that adult eclosion would shift to match any changes in the photoperiod so that it would occur during early scotophase (2005). I found that mosquito larva reared under the reverse period experienced a shift in the rhythm of adult emergence to match the photoperiod of the incubator. This suggests that these rhythms are endogenous in *Cx. pipiens* f. *molestus* and can be entrained by the photoperiod exposed to during larval development (Saunders 2002).

When the mosquitos reared in 12:12 D:L were moved into a 12:12 L:D light regime, these mosquitos maintained a rhythm that matched the photoperiod of the incubator. The maintenance of the larval photoperiod when moved to a new photoperiod is seen in other species of insects. A study on the Mediterranean flour moth, *Anagasta kühniella*, found that under natural light conditions moths kept in a reverse photoperiod during larval development experienced shifts in the rhythms of adult emergence. Like *Cx. pipiens* f. *molestus*, the moth's rhythm shifted so adult eclosion occurred during the scotophase period (Bremer 1926, as cited in Beck 1980). An earlier study found this to also be true for the intertidal midge, *Clunio marinus*. After being moved from a 12:12 D:L light regime to a 12:12 L:D light regime, the eclosion rhythm for the midges occurred during the time of the dark period they were kept in during larval

development (Neumann 1973 as cited in Beck 1980). These demonstrate how photoperiod can influence adult eclosion when kept under constant temperature conditions (Beck 1980).

Under constant conditions (i.e. complete darkness or complete light), insects display free-running rhythms. The previously mentioned studies on the intertidal midges (Neumann 1973 as cited in Beck 1980) and the Mediterranean flour moths found such results. Interestingly, the study on the Mediterranean flour moth found that moths reared under constant light appeared to become entrained to a photoperiod introduced as late as 48 hr before adult emergence (Giebultowicz and Cymborowski 1976; Moriarty 1959 as cited in Beck 1980). I found that *Cx. pipiens* f. *molestus* maintained circadian rhythms even when larval development occurred under a constant light condition. Mosquitos kept as larva under constant light had a circular mean during scotophase, and those kept in the constant dark treatment had a circular mean that was near the beginning of scotophase. I predicted that adult emergence would be arrhythmic, and these results did not match that prediction. Further study can investigate whether there was entrainment to the light cycle exposed to as pupa.

I initially postulated that inactivating mutations in the known clock genes caused the lack of diapause of *Cx. pipiens* f. *molestus*. However, I found that all known clock genes plus lark, a gene known to play a role in eclosion (Saunders 2002), were expressed in both the North American *Cx. pipiens* f. *molestus* and the European *Cx. pipiens* f. *molestus*. The basis for my original prediction was based on the knowledge that the sister taxon *Cx. pipiens* require functioning clock genes to initiate diapause (Meuti et al. 2015). Since it is believed that *Cx. pipiens* f. *molestus* arose from *Cx. pipiens* f. *pipiens* (Kothera et al. 2010), diapause is thought to be an ancestral trait and a loss of the ability to enter a diapause would be due to genetic differences. Another reason I thought that the lack of diapause was caused by an inactivating

mutation is that there is a relationship between clock genes and photoperiodic induction of diapause (Meuti and Denlinger 2013). Also, previous studies have shown that knocking out clock genes can cause aversion to diapause (Ikeno et al. 2010). Since the level of expression of the clock genes in *Cx. pipiens* f. *molestus* was unknown, the lack of diapause could be caused by an under or over expression of said genes.

A similar study done on the nondiapausing (nd) variant of the flesh fly, *Sarcophaga bullata* found that the nd variants do not undergo pupal diapause due to an elevation in the expression of *per* and *tim*. This elevated expression also caused arrhythmic adult emergence when compared to the wild type flesh flies that undergo pupal diapause. These wild type flesh flies also displayed a diel adult eclosion rhythm. The authors suggested that a disruption in the circadian clock mechanism might be the cause of the arrhythmic eclosion (Goto et al. 2006). Although a rhythm is expressed in *Cx. pipiens* f. *molestus,* it would be beneficial to look at the expression levels of the clock genes in the future in order to accurately determine the role the genes play.

It is possible that the lack of diapause in *Cx. pipiens* f. *molestus* may not be due to abnormalities in the clock genes. In *Cx. pipiens* microRNAs, which are non-coding RNAs that will regulate the expression of genes after transcription, are associated with the regulation diapause. A difference in microRNAs between *Cx. pipien* and *Cx. pipiens* f. *molestus* could influence the avoidance of diapause in *Cx. pipiens* f. *molestus* (Meuti et al. 2018). Diapause in *Cx. pipiens* is also controlled by the insulin-signaling pathway and FOXO (forkhead transcription factor). Sim and Denlinger demonstrated that they control important aspects of diapause, such as stopping ovarian development (2008). In *Cx. pipiens*, the nondiapause phenotype is promoted by long day conditions and the diapause phenotype is caused by short day

conditions. Mosquitos with the nondiapause phenotype experience the synthesis of insulin-like peptide 1, which stimulates the synthesis of JH and the suppression of FOXO. It is thought the presence of insulin will suppress the expression of FOXO. On the other hand, the insulin-signaling pathway is shutdown in mosquitos with the diapause phenotype, which leads to no JH being synthesized and the activation of FOXO (Denlinger and Armbruster 2014). Although there are no data on the role of FOXO and the insulin-signaling pathway in *Cx. pipiens* f. *molestus*, this can be an avenue of future study if it is shown that clock genes do not cause the lack of diapause in these mosquitos.

Many studies have shown that clock genes and the circadian clock play important roles in the induction of diapause in insects. However, it is still unknown why *Cx. pipiens* f. *molestus* do not undergo diapause while the above ground sister taxon *Cx. pipiens* f. *pipiens* does. Further study into the mechanical aspects of *Cx. pipiens* f. *molestus* circadian clock is needed and can lead to better understanding of why and how they avoid diapause.

Conclusions

The urban mosquito *Cx. pipiens* f. *molestus* maintains circadian rhythms despite lack of ability to enter diapause. There are no inactivating mutations in the known clock genes, and they are all expressed. Further studies should investigate the mechanical aspects of the circadian clock and to check for under or over expression of the clock genes. There is also the possibility that there is a hormonal cause for the lack of diapause. More research is needed in this area.

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