Plasmodium relictum infection in Culex quinquefasciatus (Culicidae) decreases diel flight activity but increases peak dusk flight activity

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Abstract

Parasites are recognized for their ability to modify host physiology and behaviors in ways that increase parasite fitness. Protozoan parasites of the genus *Plasmodium* are a group of widespread vector-borne parasites of vertebrates, causing disease to a wide range of hosts, but most notably to human and avian hosts. We investigated whether infection with the avian malaria, *Plasmodium relictum* (GRW4 lineage), impacts flight activity in one of their natural vectors, *Culex quinquefasciatus*, using both parasites and mosquitoes colonized from local populations in East-Central Texas, USA. Groups of *Culex quinquefasciatus* were allowed to feed directly on canaries with active *Plasmodium* infections and control canaries with no *Plasmodium* exposure history. To test how *Plasmodium* sporogenesis impacts mosquito activity behavior, engorged mosquitoes from both control and experimental trials were placed into a Locomotor Activity Monitor after oviposition and beam breaks were monitored for eight days. When analyzed by total diel activity, *Plasmodium*-infected mosquitoes had significantly reduced flight activity when compared to control mosquitoes. However, when analyzed at the peak activity hours of dawn and dusk, infected mosquitoes flew significantly more than control mosquitoes in the first 30 minutes of light to dark transition (dusk), but not in the 1-hour and 2-hour periods after the transition. Flight activity at days 14 to 17 post-*Plasmodium* infection had greater flight activity than earlier days (9 to 12 post-infection), which coincides with when *P. relictum* oocysts erupt and sporozoites travel to the mosquito salivary glands. Based on this study, avian malaria parasites increase the flight activity of these mosquitoes at hours known for peak host-seeking behavior but decrease overall diel activity. Although the ramifications of this behavioral change for *Plasmodium* transmission are unclear, results provide additional empirical evidence suggesting that avian malaria can augment mosquito behavior, and subsequently transmission potential.

Introduction

Parasite-host co-evolution has resulted in complex adaptations, many of which lead to modifications of host behavior or fitness. Parasites can be pathogenic to hosts, introducing adverse effects that cause morbidity or mortality (Christensen 1978, Beach et al. 1985, Koella et al. 2002, Valkiūnas and Iezhova 2004, Valkiūnas et al. 2014, Gutiérrez-López et al. 2019b, Yang et al. 2019, Adams et al. 2021). In other relationships, parasite infection of vertebrate hosts can result in modified behavior (Lafferty and Morris 1996), or have no observable effects on host biology (Ashby and Boots 2015). Vector-borne pathogens, which respond to adaptive pressure from both vertebrate and arthropod hosts, are no exception; parasite-induced alterations in vector behavior and biology can significantly influence parasite transmission (Hacker and Kilama 1974, Hogg and Hurd 1997, Gutiérrez-López et al. 2019b). Vectorial capacity is a framework describing all the intrinsic and extrinsic factors that contribute to an arthropod vector's ability to transmit pathogens, including survivorship, vector competence, and number of infective bites (Kramer and Ciota 2015). Parasite-induced modifications of host-seeking activity or flight activity may increase (or decrease) the number of infective bites delivered to naïve hosts or alter survivorship by augmenting the magnitude of high-risk behaviors, thus changing vectorial capacity by increasing mosquito survivorship.

Mosquito flight is a risky behavior that may lead to increased mortality in mosquito hosts. By modifying the flight behavior of its mosquito host, Plasmodium parasites can guarantee sporogenesis, and increased probability of transmission.

Although malaria parasites (*Plasmodium* spp.) are responsible for one of the most detrimental human diseases, the impact of these parasites on some aspects of their mosquito hosts remains unclear (Cator et al. 2012, Santiago-Alarcon and Ferreira 2020). Prior studies demonstrate *Plasmodium* parasites can alter mosquito survivorship, feeding behavior, and even fecundity indicating that these parasites are generally detrimental to mosquito fitness (Koella et al. 1998, Anderson et al. 1999, Koella et al. 2002, Lacroix et al. 2005, Cornet et al. 2013a, b, 2019). However, in some scenarios, these parasite-induced modifications can benefit the transmission of *Plasmodium* parasites. For example, Vezilier et al. (2012) demonstrated that although mosquito fecundity was decreased in *Culex pipiens* mosquitoes infected with *Plasmodium relictum* (lineage SGS1), mosquito survivorship was increased (Vézilier et al. 2012). This suggests that parasites divert reproductive resources within the mosquitoes to extend their survivorship.

Locomotion is fundamental to mosquito biology as they obtain nutrients required to survive and reproduce. Therefore, a reduction in flight in a mosquito may have negative consequences on their survival or reproductive fitness. Prior studies have identified a reduction in flight activity after infection with a pathogen. Berry et al (1987) found that the filarial nematode *Dirofilaria immitis* significantly reduced *Aedes aegypti* flight activity eight days after infection with the parasite. Similarly, Rowland and Boersma (1988) found flight activity in *Anopeheles stephensi* mosquitoes was reduced by approximately 33% 17 days post-infection with *Plasmodium yoelii* parasites. A variety of parasite taxa have been shown to alter mosquito flight activity, but few studies have attempted to quantify flight activity post-infection with *Plasmodium* parasites due to its challenging nature. These data enhance our ability to accurately predict disease transmission or implement vector-borne disease preventions in areas with high *Plasmodium* transmission.

The effect of *Plasmodium* parasites on the behavior of mosquito hosts has been explored in several studies with conflicting results (Santiago-Alarcon and Ferreira 2020). Koella et al. (1998) demonstrated alterations to *Anopheles gambiae* mosquito biting behavior that would improve the transmission of *Plasmodium falciparum* parasites, including an increase in multiple biting events (Koella et al. 1998). Other studies have shown that vertebrate hosts infected with *Plasmodium* spp. are more attractive to both infected and uninfected mosquitoes, suggesting that the parasites are altering the host-seeking behavior of the mosquitoes (Cornet et al. 2013a, b, Díez-Fernández et al. 2020). However, other studies have demonstrated no effect on the attractiveness of mosquitoes to *Plasmodium*-infected vertebrate hosts, or that it is dependent on the intensity of infection (Yan et al. 2018, Gutiérrez-López et al. 2019a). Modifications to mosquito-host attraction by *Plasmodium* parasites can have important ramifications for transmission and warrants further study.

Avian *Plasmodium* spp. (avian malaria) provide a model system to study the effects of parasite infection on the behavior of their mosquito hosts. Avian malaria lineages are closely related to human *Plasmodium* parasites and have a cosmopolitan distribution (Valkiūnas 2005, Bensch et al. 2009). Because of this, many studies utilize avian malaria parasites to study vector-parasite interactions to contrast relationships in multiple systems. Here we quantify the flight activity of *Culex quinquefasciatus* mosquitoes infected with the avian parasite *Plasmodium relictum* (GRW4 lineage).

Materials And Methods

Bird and Plasmodium Collection

Wild house sparrows (*Passer domesticus*) were captured during the summer of 2020 in College Station, Texas using 12-meter mist nets with 30 mm mesh size (Association of Field Ornithologists, Portland, ME). Each bird was immediately exsanguinated to recover blood for parasite isolation and molecular diagnostics. Exsanguination was carried out using a 28 ½ gauge syringe that was treated with heparin (Sagent Pharmaceuticals, Schaumberg, IL, USA) to prevent clotting. About 1–2 µl of blood was used to create two thin blood smears that were air dried, fixed in 100% methanol, and later stained with Giemsa stain (Valkiūnas 2005). The remaining blood was kept on ice and stored up to 2 at 4°C until use. The stained smears were screened in 10x10 frames at 40x magnification to determine the presence of *Plasmodium* as well as any other haemosporidians and trypanosomes. Each frame had approximately 1000 red blood cells. Screening of blood smears was done twice to ensure no false negatives. All work with wild birds was approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC AUP 2018 – 0144) and Texas Parks and Wildlife Scientific Research Permit (No. SPR-0512-917).

Mosquito Colony and Maintenance

Culex quinquefasciatus colonized in the summer of 2018 from College Station, Texas (Adams et al. 2021) were utilized for these experiments, and were between F20-25 generations removed from the wild. Mosquitoes were maintained on a natural night and day light cycle (10.5 h light, 14.5 h dark) with a constant 50% humidity at 27°C. Adult mosquitoes in the colony were maintained on commercially acquired chicken blood treated with Alsever's solution (Hemostat, Dixon, CA) and a 10% sucrose solution was provided *ad libitum*.

Plasmodium propagation

Plasmodium relictum (GRW4 lineage) obtained from wild house sparrows was amplified in domestic canaries (*Serinus canaria*) to high parasitemia (~ 10%) to feed to mosquitoes. Infected house sparrow blood (50–100 UL) was inoculated into canaries by jugular venipuncture and allowed to amplify in canaries for 10–12 days post-infection. Infection was monitored and confirmed using microscopy and PCR (Bensch et al. 2000, Valkiūnas 2005). Initial infections are usually below detection limits and required blind passages of blood to two additional canaries on day 7 post-exposure. Canaries were routinely screened via microscopy on days 4 and 7. If canaries had not cleared the infection by day 10,

canaries were exsanguinated, and blood was cryopreserved for future study. Canaries that cleared infection were euthanized and were not utilized again for passaging. The *Plasmodium* used for the current study was passaged between 2–3 canaries from the wild sparrow before exposure to mosquitoes. All work with captive canaries was approved by Texas A&M University Institutional Animal Care and Use Committee (AUP 2018 – 0175)

Mosquito infection

Four replicates of mosquito infection were conducted given the constraint on the number of individual mosquitoes that could be monitored for flight activity at a given time. In each trial, cohorts of 100 one week old female *Cx. quinquefasciatus* were offered either a canary between 6–8 days post-infection with *P. relictum* (GRW4 lineage) or a control canary that had never been exposed to *Plasmodium* (verified using PCR and microscopy). Feeding events occurred between 5-7am as prior work with these colonized *Cx. quinquefasciatus* determined that this period yields the highest blood feeding success. To do this, canaries were restrained with flexible athletic bandage over a container with mesh for mosquito feeding for up to 30 minutes (Fig. 1).

Mosquitoes were then knocked down on ice, and only fully engorged females were removed and added to a separate container (plastic 16cm by 21cm cup with mesh lid) with other engorged females (Sella 1920). The blood engorged mosquitoes were provided 10% sucrose solution (*ad libitum*) and allowed to develop eggs. A small cup containing water was placed into the cage on day 6 post-feeding and retrieved on day 8. On day 8, mosquitoes were knocked down and 16 of those that had laid eggs were individually removed and placed into glass tubes for activity monitoring in the *Drosophila* Locomotor Activity Monitor 25 (LAM 25) (TriKinetics Inc, Waltham, MA). Each tube had a cotton ball on one end to prevent escape, and a 10% sucrose solution on the other (Fig. 1). The activity monitor houses 32 mosquitoes at once (8 columns by 4 rows), so 16 mosquitoes exposed to *P. relictum* and 16 mosquitoes fed on control birds were simultaneously examined. Treatments were spaced so that they would occupy every other row (ie. 8 infected, 8 controls, etc.). Mosquitoes were then left in the activity monitor inside an environmental chamber for 10 days undisturbed at 56% humidity, 27°C, and a light-dark cycle of 12 hours light followed by 12 hours of dark (19.3 cu ft. Peltier Refrigerated Incubator, Shel Lab, Cornelius, OR). On day 10, mosquitoes were removed, and *Plasmodium*-exposed individuals were dissected for midguts and salivary glands. Midguts were stained with 0.05% mercurochrome solution for identification of oocysts at low magnifications, and subsequently preserved for DNA extraction of oocysts and midguts (Valkiūnas 2005). Salivary glands were added to a microscope slide containing a drop of phosphate buffer solution, erupted using dissecting probes, and allowed to dry (Valkiūnas 2005). Using the same method as blood smears, salivary glands were stained and sporozoites were identified under 100x oil immersion. Heads and thoraces were preserved for DNA extraction of sporozoites.

Molecular diagnostics and sequence determination

DNA from infected mosquito heads/thoraces and midguts was extracted to detect the presence of sporozoites and oocysts respectively. This was done following the Bio-tek E.Z.N.A. (Omega, Norwalk, CT)

manufacturer recommendations for tissue extraction with slight modification; blood samples incubated at 70°C for a minimum of one hour. Polymerase Chain Reaction (PCR) was run on all samples to confirm infection in thoraxes and midguts using HAEMF and HAEMR2 primers by amplifying a 478 bp region of the cytochrome b gene following previously published thermocycling parameters (Bensch et al. 2000). The same method was used for identification of parasite in bird blood, except DNA from blood was extracted using the Bio-tek E.Z.N.A. blood extraction protocol. Parasite lineage determination was done by Sanger sequencing in forward and reverse directions (Eton Biosciences Inc., San Diego, CA). Clean sequences were queried using GenBank and MalAvi databases to identify similar sequence matches.

Data Analysis

Mosquito activity was only analyzed for days 2 through 9 (days 9 through 16 post-infection) of activity monitoring in the environmental chamber (Fig. 2). Day 1 was provided to the mosquitoes to acclimate to the new 12:12 day-night cycle in a new incubator, and day 10 data was removed to allow for an even number of analysis days in the code. Mosquito activity was recorded at 30 second intervals and combined into 30-minute increments for analysis consistent with prior research utilizing this locomotor activity monitor (Chiu et al. 2010, Eilerts et al. 2018). Given that *Cx. quinquefasciatus* infection rates with *Plasmodium* are between 80–95% (Vézilier et al. 2010), we only analyzed data from females that were confirmed to be infected with *Plasmodium*, either molecularly or by sporozoite microscopy post activity monitoring. The uninfected control mosquitoes included only those that fed on the uninfected canary. Any mosquitoes (infected or control) that died during flight activity recording at any time during the 10 days were removed from analysis. Mann-Whitney U-Tests were utilized to analyze data as a non-parametric alternative to t-tests to compare the mean flight activity of infected and control mosquito populations due to data containing numerous no-flight events (i.e., zeros). Mann-Whitney U-Tests were also utilized to compare early and late *P. relictum* infected mosquitoes to quantify the effect of sporozoite entry into the mosquito salivary glands. Early infection mosquito data were days 1 through 4, and late infection mosquito data were days 5 through 8 (Fig. 2). Analyses were conducted on both total flight activity and peak activity hours since visible differences in activity were observed at the photoperiod transition events (e.g. photophase to scotophase) (Fig. 3). Peak activity analysis was conducted on 2-hour (ZT 0–2, 12–14), 1-hour (ZT 0–1, 12–13), and 30-minute (ZT 0-0.5, 12-12.5) intervals for analysis, as well as photophase (ZT 0–2) and scotophase (ZT 12–14) transition (i.e. dawn and dusk) hours. We used a two-hour period after light transitions for analysis due to increased diel activity for this mosquito species. Zeitgeber time (ZT) is frequently utilized in circadian rhythm analysis as a description of when lights are turning on and off. Here ZT 0 represents 7:30 am when lights turn on (dawn), and ZT 12 represents 7:30 pm when the lights turn off (dusk) (Fig. 3). Figures were created using the Rethomics framework (Geissmann et al. 2019). Analyses were performed using the R statistical software v3.5.2 (R Foundation for Statistical Computing, Vienna, Austria). Means are presented ± standard error, and we used an alpha value of $p = 0.05$ for judging statistical significance.

Results

The collection of Plasmodium infected birds

Twenty-one house sparrows were exsanguinated on June 15, 2020 in College Station, Texas, USA. Only one of the birds (Male) tested positive for Haemosporida infection via microscopy and PCR. After sequencing and consensus sequence was generated, the closest match in the NCBI BLAST database was a 99.58% match to sequence MG018687.1 which is *Plasmodium relictum* (GRW4).

Infected vs control flight activity comparison

When comparing total flight activity (i.e. 24 hours across 8 days), a statistically significant difference in total diel flight activity was observed between the control and *Plasmodium*-infected mosquitoes (Mann-Whitney U-Test, df = 1, W = 121601679, $p < 0.001$). Control mosquitoes had a mean flight activity of 3.13 ± 0.15 beam breaks throughout the ten days of observation, and infected mosquitoes had a mean flight activity of 3.10 ± 0.13 beam breaks (Table 1). When looking specifically at peak activity during both photophase and scotophase transitions, no statistically significant difference was observed for both 2-hour (ZT = 0–2, 12–14; Mann-Whitney U-Test, df = 1, W = 3412809, $p = 0.2558$) and 1-hour (ZT = 0–1, 12–13; Mann-Whitney U-Test, df = 1, W = 882151, $p = 0.402$) crepuscular periods. However, when analyzed by a 30-minute interval of both photophase and scotophase transitions (ZT = 0.5, 12.5), *Plasmodium*-infected mosquitoes had statistically significant increased flight activity (Mann-Whitney U-Test, df = 1, W = 249449, $p < 0.001$). When looking only at scotophase transition peak activity (ZT 12–14), a statistically significant difference in activity between mosquito treatments was observed in the 1-hour (ZT = 12–13; Mann-Whitney U-Test, df = 1, W = 71008, $p < 0.001$) and 30-minute analyses (ZT = 12-12.5; Mann-Whitney U-Test, df = 1, W = 232738, $p = 0.019$), but not the 2-hour analysis (Mann-Whitney U-Test, df = 1, W = 859735, $p = 0.714$). When looking at only photophase transition activity activity (ZT 0–2), no statistically significant difference was found in any of the 2-hour (Mann-Whitney U-Test, df = 1, W = 853423, $p = 0.3468$), 1-hour (Mann-Whitney U-Test, df = 1, W = 213620, $p = 0.629$), and 30-minute (Mann-Whitney U-Test, df = 1, W = 55166, $p = 0.674$) analyses.

Table 1

The mean mosquito flight beam breaks for multiple time intervals comparing *Plasmodium relictum* infected and control *Cx. quinquefasciatus*. Peak crepuscular activity refers to the mean of both photophase and scotophase transition times combined (i.e. dawn and dusk) and corresponds to ZT 0–2 and 12–14. For the scotophase transition period analysis, only peak activity during scotophase (ZT 12–14) were analyzed, not including the photophase transition (ZT 0–2). 2-hour analysis corresponds with ZT 0–2 (Photophase transition) and ZT 12–14 (scotophase transition). 1-hour analysis corresponds with ZT 0–1 and 12–13. 30-minute analysis corresponds with ZT 0-0.5 and 12-12.5. Forty-seven mosquitoes were analyzed for both infected and control groups. Activity means are presented as mean ± standard error.

	***P. relictum* (+) (n = 47)**	**Control (-) (n = 47)**
	Mean	Mean
Total Activity	3.096 ± 0.129	3.125 ± 0.149
Peak Crepuscular (ZT 0–2, 12–14)		
2 hr	12.38 ± 0.63	9.32 ± 0.41
1 hr	23.24 ± 1.04	17.25 ± 0.64
30 min	39.58 ± 1.68	26.94 ± 0.94
Scotophase Transition Period (ZT 12–14)		
2 hr	18.30 ± 1.11	12.84 ± 0.69
1 hr	33.59 ± 1.71	22.98 ± 0.95
30 min	53.70 ± 2.64	31.31 ± 1.28

Early vs Late Plasmodium relictum infection

When comparing total flight activity (i.e. 24 hours across 4 days), of early and late *P. relictum* infected mosquitoes a statistically significant difference in total diel flight activity was observed (Mann-Whitney U-Test, df = 1, W = 23161844, $p < 0.001$). Early infection mosquitoes had a mean flight activity of 2.41 ± 0.16, and late infection mosquitoes had a mean flight activity of 3.78 ± 0.20 (Table 2). When looking specifically at peak activity time of early and late *P. relictum* infected mosquitoes, no significant difference was observed in either the 2-hour (ZT = 0–2, 12–14; Mann-Whitney U-Test, df = 1, W = 654529, $p = 0.488$) and 1-hour analysis (ZT = 0–1, 12–13; Mann-Whitney U-Test, df = 1, W = 157482, $p = 0.120$), however the 30-minute analysis was statistically significant (ZT = 0-.5, 12-12.5; Mann-Whitney U-Test, df = 1, W = 35321, $p < 0.002$).

Table 2
The mean mosquito flight beam breaks for multiple time intervals comparing early and late *P. relictum* infected *Cx. quinquefasciatus*. Peak crepuscular refers to both photophase and scotophase transition times (i.e. dawn and dusk). 2 hour periods analysis periods corresponds to ZT 0–2 for photophase and 12–14 for scotophase. 47 mosquitoes were analyzed for both early and late infection. Activity means are presented as mean ± standard error.

	Early (Days 1–4)	**Late (Days 5–8)**
	Mean	Mean
Total Activity	2.412 ± 0.161	3.780 ± 0.201
Peak Crepuscular		
2 hr	10.57 ± 0.83	14.19 ± 0.94
1 hr	19.51 ± 0.16	26.98 ± 0.20
30 min	33.17 ± 1.95	45.99 ± 2.69

Discussion

We evaluated the ability of avian malaria lineage *P. relictum* (GRW4) to modify the flight activity of a primary avian malaria vector, *Cx. quinquefasciatus*. Our results demonstrate that *P. relictum* infection in *Cx. quinquefasciatus* mosquitoes decreases overall diel flight activity of the mosquitoes but increases activity in their peak activity hours (Fig. 3). The increased flight activity due to *Plasmodium* infection was most pronounced during the 30min period following the transition from light to dark (i.e. dusk). *Culex* mosquitoes are known to be crepuscular in host-seeking behavior (Yee and Foster 1992). The increase in flight activity around dusk may translate to increased host-seeking activity and contact with birds, which could increase transmission of *Plasmodium*. Other studies have demonstrated alterations to mosquito host-seeking and feeding behavior including increases in host-attractiveness to *Plasmodium*-infected mosquitoes and multiple biting events (Koella et al. 1998, Cornet et al. 2013a, b, Díez-Fernández et al. 2020). Similar studies have evaluated infection of mosquitoes in different pathogen systems and have also identified modifications to flight activity. This means that parasite alteration of mosquito flight activity behavior is not isolated to *Plasmodium* mosquitoes and may be widespread across parasitism (Berry et al. 1987a, Berry et al. 1987b, Rowland and Boersma 1988).

We quantified the flight activity of *Cx. quinquefasciatus* mosquitoes in an activity monitor that detects beam breaks for every flight event as a proxy for host-seeking behavior, but this approach has limitations. Flight activity at any given time can contribute to multiple behaviors including host-seeking, oviposition seeking, or sugar source-seeking. We attempted to eliminate one of these behaviors by only analyzing

mosquitoes that laid eggs prior to quantification of flight activity, but this does not explicitly mean the mosquitoes were host-seeking. We observed a decrease in the total diel activity with *Plasmodium* infection which could be the result of diverted resources within the mosquito. However, we observed *Plasmodium* infection significantly increased flight activity shortly following the light to dark transition which would increase energy expenditures during this crepuscular period, potentially because of *P. relictum* manipulation. Other studies have also demonstrated *Plasmodium* parasites diverting resources to facilitate further transmission of the parasites. Vézilier et al (2012) also found that *Cx. quinquefasciatus* mosquitoes infected with *Plasmodium relictum* (SGS1 lineage) had increased survivorship, but decreased fecundity, which could serve the purpose of furthering the parasite transmission (Vézilier et al. 2012). A decrease in overall *Cx. quinquefasciatus* flight activity post-infection benefits the *P. relictum* pathogen as it can complete sporogenesis and potentially infect another avian host.

In our study, we were able to quantify an increase in activity between early and late *Plasmodium relictum* infected mosquitoes. Day 5 of activity monitoring correlated with Day 12 post-infection, which is generally accepted as within the range of time (7–16 days post-infection) where *Plasmodium* oocysts erupt, and sporozoites travel to the mosquito salivary glands (LaPointe et al. 2012, Valkiūnas et al. 2015). On day 5 of our analysis, we saw an overall increase in flight activity (total and peak) (Fig. 3), which is consistent with higher sporozoite infection levels in mosquitoes. This behavioral pattern may be suggestive of parasite manipulation as overall decreased flight activity may optimize *Plasmodium* transmission by permitting mosquitos to develop sporozoite infections in the salivary glands before partaking in high-risk host-seeking behaviors. This behavioral modification would limit this risk of death to the mosquito by more rigorously host-seeking during a shorter interval and increase the parasites probability of infecting new hosts.

Alternatively, behaviors identified here may be due to alterations in mosquito tolerance to light changes. Multiple studies have removed the first two hours of mosquito activity data after light transitions due to the “startle response” that occurs when mosquitoes experience light change in artificial settings (Lima-Camara et al. 2011). This response to the changing light was also observed in the current study, although from field studies *Cx. quinquefasciatus* is also considered crepuscular (Niebylski and Meek 1992). This response to changing light might have increased activity via a startled respons, but the *P. relictum* positive individuals had a 1.71-fold higher flight activity in the 30-minute period following light transition from photophase to scotophase, suggesting some effect on flight behavior by *P. relictum* (GRW4) infection.

The measure of flight activity recorded with the instrument in this study is not a direct measure of host-seeking behavior. Prior studies in the avian malaria system have found that *P. relictum* infected and uninfected mosquitoes were highly attracted to *P. relictum* infected birds, supporting the claim that mosquito host-seeking behavior is altered by this parasite (Cornet et al. 2013a, b, Díez-Fernández et al. 2020). Additionally, a study by Lacroix et al. (2005) in the human malaria system found similar results (Lacroix et al. 2005). However, one study by Lalubin et al. (2014) found that there was no evidence of this

alteration, and perhaps a parasite avoidance by these mosquitoes (Lalubin et al. 2014). Our data support a modulation in flight activity from less overall flight activity to increased flight activity at peak host-seeking times, which could lead to an increase the transmission of this *P. relictum* parasite. The observations of this study warrant further host choice experiments to see if *P. relictum*-infected *Culex* mosquitoes increase contact and feeding on birds in a controlled setting.

Overall, our study observes an effect of *P. relictum* infection on the flight activity of *Cx. quinquefasciatus* mosquitoes during crepuscular hours. To our knowledge this is the first study to quantitatively evaluate the effect of avian malaria on *Culex* mosquito flight behavior. We did this using a natural system combining *P. relictum* (GRW4) and its vector, *Cx. quinquefasciatus*, colonized from the same study location of College Station, Texas (Adams et al. 2021). Although these results provide further evidence that *Plasmodium* infection can modify vector behavior, further work is needed to determine if increases in flight activity during peak hours of host-feeding promote parasite transmission. Future studies will attempt to understand why *P. relictum* decreases the total flight activity of *Cx. quinquefasciatus* mosquitoes, and how these behavioral modifications might impact vector-control strategies.

Declarations

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Author Contribution

DRA, AJG, JM, MAS, GLH designed the experiments. DRA and GLH collected data. DRA analyzed data. DRA wrote the manuscript. All authors significantly contributed to the improvement of this manuscript.

Animal Ethics Declaration

We certify that all animals used in this study were treated ethically with approval by the Texas A&M University Institutional Animal Care and Use Committee (IACUC AUP 2018-0144).

Funding Declaration

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Conflict of Interest

The authors declare that they have no potential conflicts of interest.

Availability of Data and Materials

The datasets supporting the conclusions of this article are available in the Texas A&M Oaktrust repository, https://hdl.handle.net/1969.1/195854.

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Figures

Figure 1

(A) Mosquitoes were kept alive for 10 days in a glass tube placed into the Drosophila Locomotor Activity Monitor 25. On one end, plastic test-tubes were used to hold the 10% sucrose solution and a sugar wick. The tubes were held in place with multiple layers of parafilm for a tight fit. On the other end, a cotton ball was used to keep the mosquito within the glass tube. Careful attention was paid to prevent the wick and cotton ball from extending too far into the glass tube so that erroneous recordings were not collected. (B) Restrained blood feeding of female *Culex quinquefasciatus* on a domestic canary. Athletic bandage was used as a restraint. Canaries were restrained for up to 30 minutes.

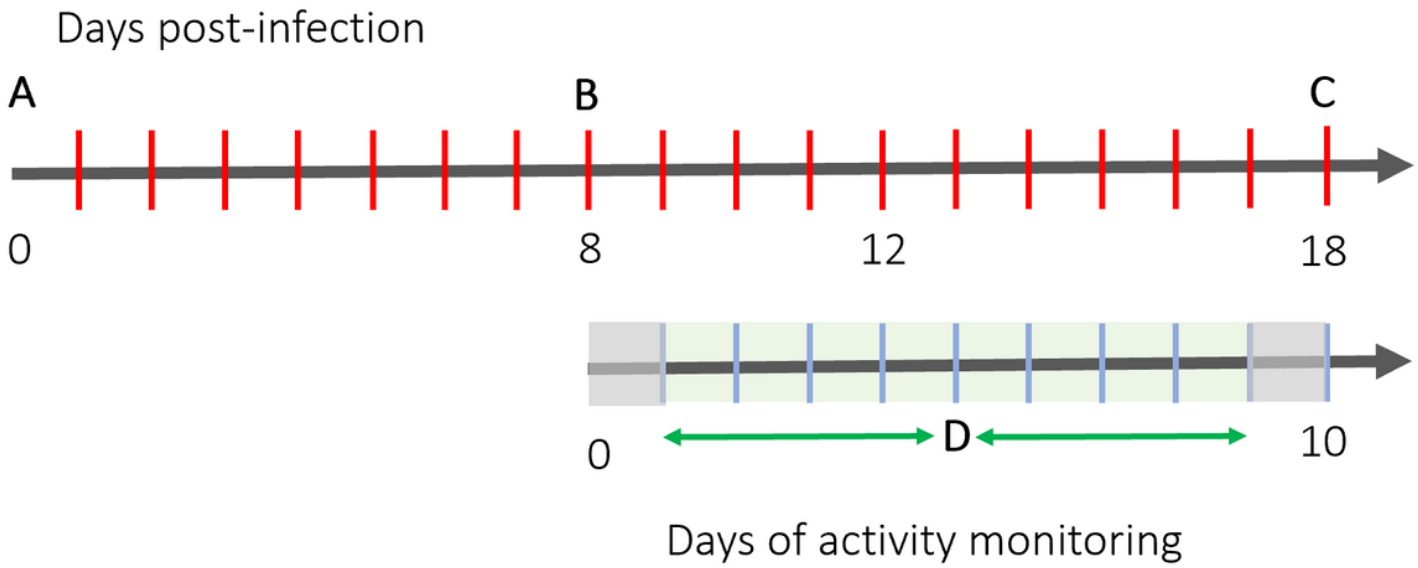

Figure 2

An experimental timeline of activities during experimentation. The top line refers to the post-infection days of *Cx. quinquefasciatus* with *P. relictum* (18 days total). The bottom line refers to the days that mosquito activity was being recorded in the environmental chamber (10 days total). Gray shaded days were removed from analyses, and green shaded days were included. Letters represent points of interest as follows. (A) mosquitoes were infected with P. relictum, (B) mosquitoes that laid eggs were added to the activity monitor, (C) all mosquitoes were removed from the activity monitor and dissected, (D) the midpoint separating early and late day analyses relating to the timing of sporozoite entry into the mosquito salivary glands. Day 12 post-infection corresponds with when we expect to see sporozoite presence in mosquito salivary glands.

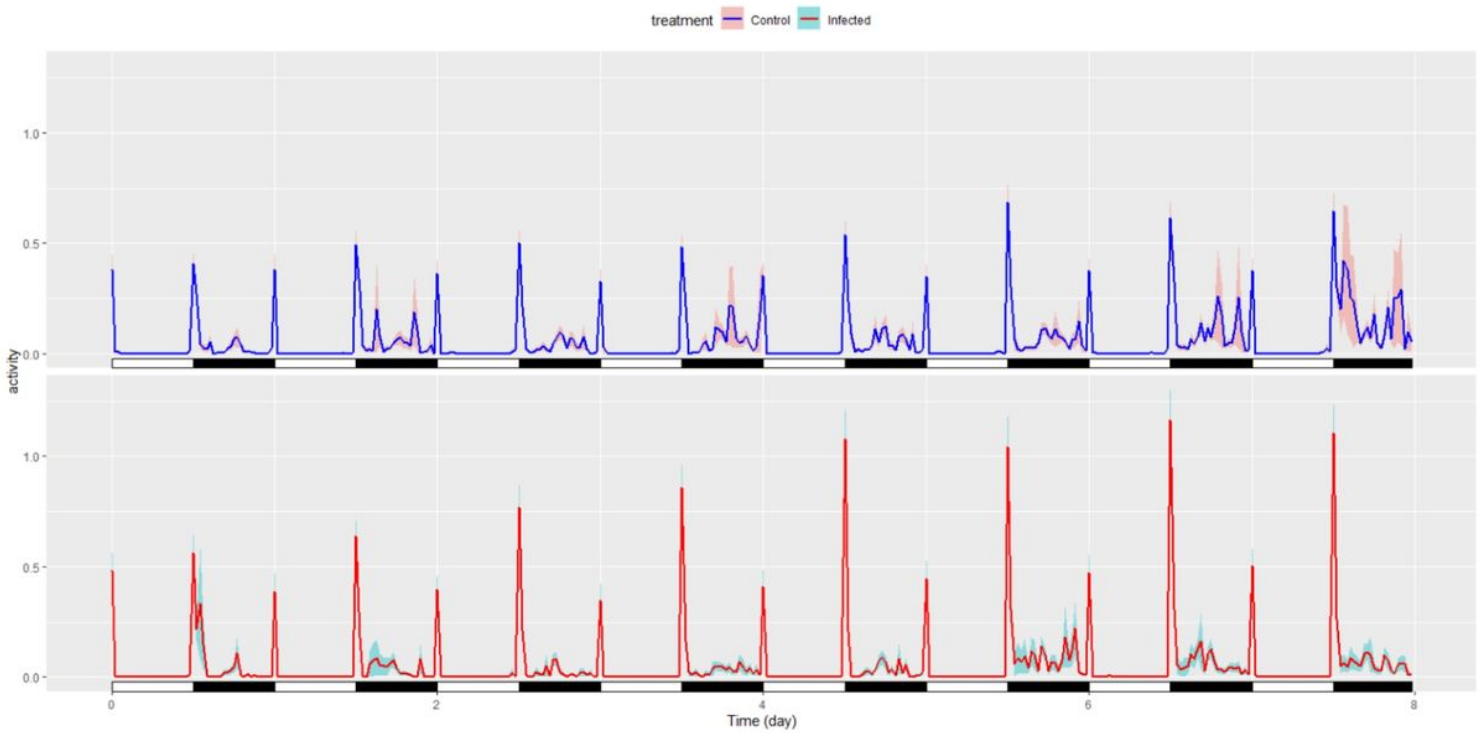

Figure 3

Activity of *P. relictum* infected and uninfected control *Culex quinquefasciatus* mosquitoes. Black and white bars represent scotophase and photophase, respectively, and combined, they represent a single day. Activity is represented by average activity during a particular time interval binned by 30-minute increments with 95% confidence intervals depicted as lightly shaded colors. 47 mosquitoes were analyzed for infected mosquitoes and 47 mosquitoes were analyzed for control mosquitoes.