IRON TOLERANCE IN BLOOD-FEEDING VS. OBLIGATE NON-BITING POPULATIONS OF THE PITCHER-PLANT MOSQUITO, *WYEOMYIA SMITHII*

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Title: Iron Tolerance in Blood-Feeding vs. Obligate Non-Biting Populations of the Pitcher-Plant Mosquito, *Wyeomyia smithii*

Mosquitoes have been called the deadliest animals on earth because they are vectors for egregious diseases such as malaria, dengue, Zika virus, and many others. As insecticide resistance becomes more prevalent and climate change-induced habitat expansion allows disease vectors to move into new areas, current vector control programs are becoming less and less effective. Female mosquitoes transmit disease during the act of taking a blood meal. Blood has the potential to be toxic due to its high heme iron content and can be lethal when ingested by male mosquitoes. The pitcher plant mosquito, *Wyeomyia smithii,* is fully interfertile throughout its geographic distribution, but northern females are obligate non-biters while southern females have the potential to take a blood meal. Prior research has shown that increasing concentrations of iron are increasingly toxic for juvenile mosquitoes (larvae) in populations of *W. smithii* whose adult females (the actual stage that bites) are either obligate non-biters or capable biters (Rasmussen 2019). The purpose of my research was to find out whether biting propensity and/or sex affect longevity in adult *W. smithii* mosquitoes when fed on carbohydrates laced with various concentrations of iron, and, additionally, whether iron affects fecundity, fertility, or net reproductive success in these populations. Using the two highest concentrations of ferrous sulfate that were toxic to larvae, I found that spiking adult food with iron sulfate solutions did not significantly affect adult longevity, fertility, fecundity, or net reproductive success, and there was no reduction in longevity of either sex. Consequently, higher concentrations of iron or other iron or metal salts would provide more promising avenues to adult mosquito control.

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Introduction

Mosquitoes have frequently been called the deadliest animals on earth (Armbruster 2018) because they are vectors for harmful diseases such as malaria, filariasis, dengue, yellow fever, and Zika viruses (Murugan et al. 2018). The need for methods of reducing infection is ever increasing, as these diseases (particularly dengue) have become steadily more prevalent during the past 50 years (Tsujimoto et al. 2018). Furthermore, arboviruses such as West Nile, Japanese encephalitis, and chikungunya have considerably expanded their geographic ranges and are appearing in new locations (Hall-Mendelin et al. 2010). This expansion is due to climate change-induced habitat suitability for disease-carrying mosquitoes, as well as globalization of trade and increasing international travel (Elbers et al. 2015).

The mosquitoes which spread diseases to humans are necessarily female, because only female mosquitoes take a blood meal (hereafter bite). Without taking a blood meal, mated females may lack the necessary protein for egg development (Nikbakhtzadeh et al. 2016). Although protein-rich blood is necessary for reproduction in many mosquito species, it has the potential to be toxic in large doses due to the high presence of iron (Tsujimoto et al. 2018). Biting is therefore metabolically costly because the mosquito must be able to sequester and transport toxic components of blood in order to reap the benefits of ingesting the blood for egg development and survival. By the end of the first reproductive cycle (including biting, digestion of the blood meal, and ovarian maturation and oviposition), 87% of heme iron from the blood meal is excreted, 6% is incorporated in the female body, and 7% is allocated to the eggs (Zhou et al. 2007). Female mosquitoes possess inside their mid-gut a membrane-like mesh

(peritrophic matrix) that sequesters heme and iron released from hemoglobin in ingested blood, thus reducing heme and iron-induced protein degradation and cell death throughout the mosquito body (Whiten et al. 2018).

Prior to taking a blood meal, adult mosquitoes have only a rudimentary peritrophic matrix (Dinglasan et al. 2010), which develops and thickens in response to the quantity of blood ingested (Baia-da-Silva et al. 2018). Female mosquitoes have the ability to sequester and rapidly excrete iron through the heme-binding mechanism of the peritrophic matrix; male mosquitoes possess a less elaborate peritrophic matrix and are much more susceptible to iron toxicity. Blood feeding is lethal for male mosquitoes when consuming about 10% as much blood as an engorged female, reducing male survival to only a few days, compared to over a month when fed only sugar (Nikbakhtzadeh et al. 2016).

Current vector control methods often rely heavily on the use of pesticides to target disease-spreading mosquito populations. Consequently, insecticide resistance is becoming a growing issue globally, and, in many countries, has led to failure of disease-control programs (Naqqash 2016). Toxic sugar-baited traps are a common vector control method used to kill both female and male mosquitoes, using attractive sugar laced with pesticides (Fiorenzano et al. 2017). Concerns about insecticide resistance and environmental effects have caused researchers to investigate environmentally benign replacements for toxic pesticides used in attractive toxic sugar baits (Revay et al. 2015). My research aims to find out the concentrations of ingested iron that are toxic to adult pitcher-plant mosquitoes, *Wyeomyia smithii*, and how this information can be applied to the development of environmentally benign vector control methods.

When considering the effects of iron on mosquitoes, *W. smithii* is an ideal study organism. This species is unique in that populations of individuals are obligate non-biters in the northern extent of their range and are capable biters in the southern portions of their range; yet, all populations remain completely interfertile. Prior research has shown that increasing concentrations of iron are increasingly toxic for juvenile mosquitoes (larvae) in populations of *W. smithii* whose adult females (the actual stage that bites) are either obligate non-biters or capable biters (Rasmussen 2019). Additionally, iron toxicity is higher for larvae of northern (non-biting adults) populations than for southern (biting adults) populations across the same range of larval iron concentrations (Rasmussen 2019). However, it is not known what levels of iron may be toxic or fatal to adult males vs. females across biting vs. non-biting populations. Determining this relative toxicity is the topic of my research.

I expected to find that iron would be more toxic to obligate non-biting populations of *W. smithii* than biting populations. I also expected that iron would be more toxic to non-biting males than biting females. In fact, I found that solutions of iron sulfate toxic to larvae did not reduce adult longevity or reproductive success in non-biting relative to biting populations or reduce longevity in adult males relative to females.

Background

The pitcher plant mosquito *Wyeomyia smithii* oviposits into and completes its pre-adult life cycle only in the water-filled leaves of the carnivorous plant *Sarracenia purpurea* (Bradshaw and Holzapfel 2017). Its range extends from the Gulf of Mexico to central Canada, and populations of this organism remain fully interfertile (Borowczak 2017). Northern populations of *W. smithii* are obligate non-biters, while southern populations have the potential to bite. This species is the only known contemporary mosquito which bites in part of its range and is obligate non-biting in the remaining part of its range (Bradshaw et al. 2018).

Figure 1: Adult *Wyeomyia smithii* descending into a *Sarracenia purpurea* leaf.

Materials and Methods

In my experiment I used four populations of *Wyeomyia smithii*, two of which are obligate non-biting Northern populations (WI, ME) and two potentially biting Southern populations (FL, AL). Populations were collected from each location during the summer of 2016.

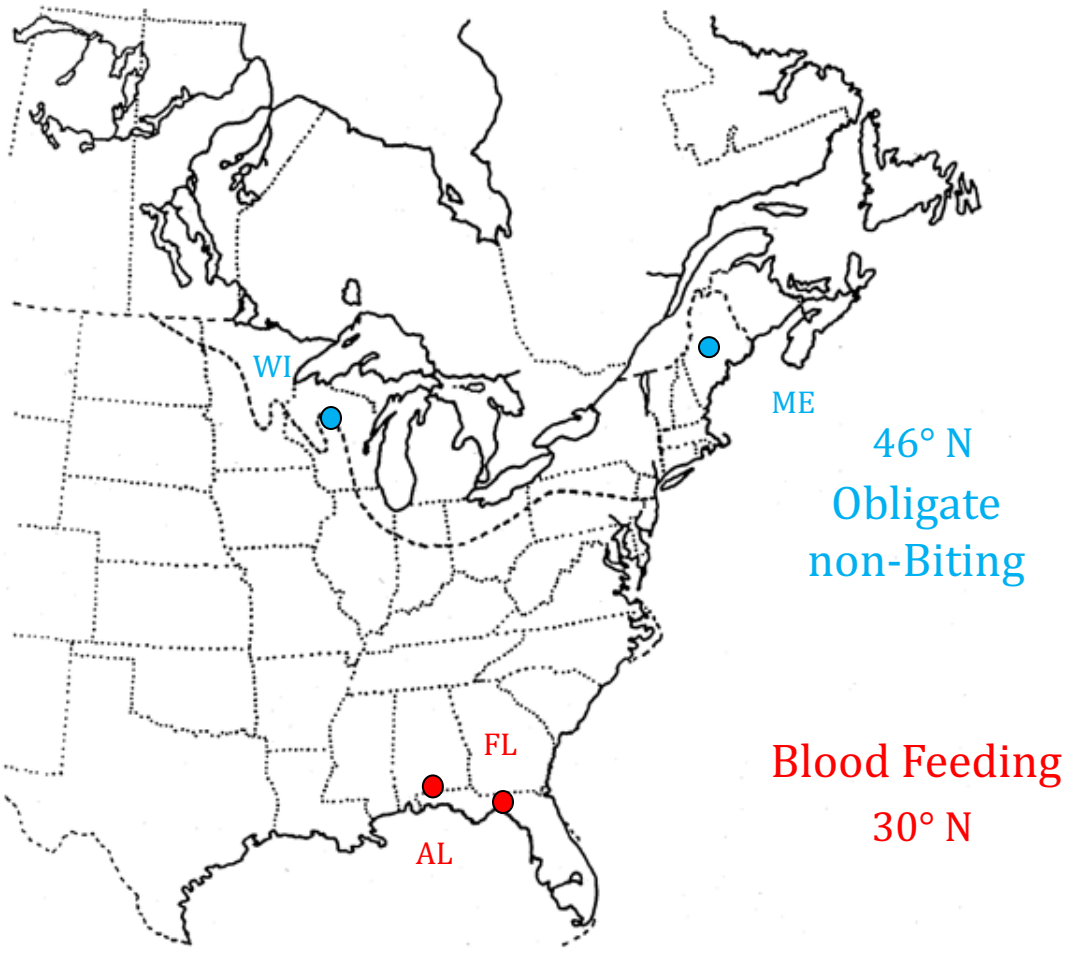

Figure 2: Origin of experimental populations. The dashed line represents the maximum extent of the Laurentide Ice Sheet.

Standard Laboratory Rearing Procedure

Populations are maintained by rearing under short day conditions (8 hours of light and 16 hours of darkness each day at 21°C) in order to induce larval dormancy (diapause). Larvae are kept in 150 mm dishes containing 35 larvae each and with 75 mL 1.08 x 10^{-1} g/L tetracycline deionized water (hereafter, tetracycline water). Larvae are fed a 4:1 mixture of Forti-Diet Pro Health guinea pig food (Kaytee©) and freeze-dried brine shrimp (San Francisco Bay Brand©), ground and sifted through a 0.5 mm filter. This sifted mixture is then mixed with tetracycline water as a slurry fed to larvae ad lib. weekly.

To stimulate development, larvae are reared under long day conditions (18 hours of light and 6 hours of darkness daily) at 92% relative humidity and a sine-wave temperature cycle ranging from 15 to 32°C each day. Up to 50 pupae are collected three times per week in 80 mL of deionized water. After three to five days, pupae are placed into adult cages containing layered 100% recycled paper towels (Natural Value™) covered by a layer of Whatman 3MM © chromatography paper, all saturated with deionized water. One *Sarracenia purpurea* leaf in 120 mL deionized water is provided to stimulate oviposition. The top and sides of the cage are lined with fiberglass window screening to permit ventilation. Five organic, pesticide-free raisins are kept on top of each cage as a carbohydrate source for the adult mosquitoes and are changed weekly. Eggs are collected three times weekly and are stored on deionized water in 150 mm dishes. Eggs are kept in short day conditions, and five days after egg collection the hatched larvae are transferred in groups of 35 to 150 mm dishes containing tetracycline water and fed a 2 mL slurry of larval food ad lib., as above.

F_0 and F_1 populations

To obtain my F_0 populations, I removed 900 diapausing larvae each from the ME, WI, Fl, and Al stock populations. I used the standard laboratory procedures described above to rear both my F_0 and F_1 generations. My experimental population sub-sets contained the same proportions of larval dishes from each date as the original stock population (dates are determined by the date of egg collection for the larvae in the dish). After rearing these larvae to adulthood and collecting their offspring, I kept 20 dishes from each of these F_1 generations, again with proportional dates in reference to the F_1 populations. I reared these larvae to adulthood and kept the first 750 F_2 offspring from each population to use as my experimental populations.

Ferrous Sulfate Solutions and Feeding Treatments

I used three treatments per population of adult mosquitoes, administered through raisins (the carbohydrate source for adult nutrition) provided to each cage. One cage per population received raisins soaked in the control deionized water treatment (0 mg ferrous sulfate/L), one received raisins soaked in deionized water containing 13 mg ferrous sulfate/L, and the remaining cage received raisins soaked in 130 mg ferrous sulfate/L deionized water. These concentrations of ferrous sulfate correspond to the highest, non-saturating solutions used by Rassmussen (2019). The treatments were labeled 0, 1, and 2, respectively, and cages were labeled with the population's acronym to coordinate my populations with populations sampled from specific localities over several decades by this laboratory (FL = locality WI; AL = locality LI; ME = locality KC; WI = locality ML) and the treatment number (ex: KC0, KC1, and KC2). I created the iron solutions using Nature Made © 325 mg ferrous sulfate tablets (65 mg iron per

tablet) dissolved into deionized water (0 tablets used for control solution, 6 tablets used for solution 2 dissolved into 3.0 L deionized water, and 300 mL of solution 2 added to 2.7 L of deionized water for solution 1). Solutions were stored in sealed 3.8 L gallon plastic jugs in a 3°C refrigerator.

Desiccated raisins were submerged in each solution two days prior to a scheduled Sunday/Wednesday/Friday change of treated raisins for each cage. Treated raisins were stored in a 3°C refrigerator, kept in three 150 mm dishes labeled 0, 1 and 2, and filled with 75 mL of their respective treatment solutions. Unused raisins were discarded.

Experimental Procedures

I collected the F_2 hatch from each population and kept 50 larvae in 150mm dishes filled with 75 mL tetracycline water and ad lib. larval food slurry on a Sunday/Friday/Wednesday feeding schedule, with larvae moved to new dishes on every other feeding day. Pupae were collected every other day, and they were assigned in a successive rotation to the 0, 1, and 2 treatments within each population. Date of eclosion and sex were recorded for each pupa. Eggs were counted Sunday/Wednesday/Friday for each cage and saved on deionized water. Five days following egg collection, hatched larvae were counted. Date of death and sex were recorded for adult mosquitoes from every cage. This process was continued until all larvae had pupated, all pupae had eclosed, and all adults had died.

Due to issues with humidity in the room with adult cages that affected one specific area of the room, adult mortality was high in the Wisconsin control group (WI0) and scored as missing data in subsequent statistical analyses.

Statistical Analyses

Adult longevity was calculated as mean day of adult death minus mean day of adult eclosion for each sex for each treatment and each population. A nested ANOVA was used to test variation among ferrous sulfate treatments, between biting (southern) vs. non-biting (northern) populations within treatments, and between sex within populations. Prior to ANOVA, frequency of hatched eggs (hereafter fertility) was arcsine transformed to approximate a normal distribution. Fecundity (eggs per eclosed female), adult longevity, and net reproductive success (hatch per eclosed female) were log10 transformed prior to ANOVA. ANOVAs were run using R Studio Version 1.2.5033 (stats package v3.6.2). F-values were calculated as mean square for a treatment level divided by mean square of the next lower nested treatment level (Sokal and Rholf 1995, Box 10.1). P-values <0.05 were scored as significant. Figures were created using R Studio packages ggplot2 and tidyverse.

Results

Adult longevity was greater in biting than non-biting populations but did not differ among ferrous sulfate treatments or between males and females (Figure 3). Non-biting populations achieved higher fecundity and net reproductive success than did biting populations, but neither fecundity nor net reproductive success differed among iron sulfate treatments within biting propensity (Figures 4, 5). Fertility did not vary between biting and non-biting populations or among ferrous sulfate treatments (Figure 6).

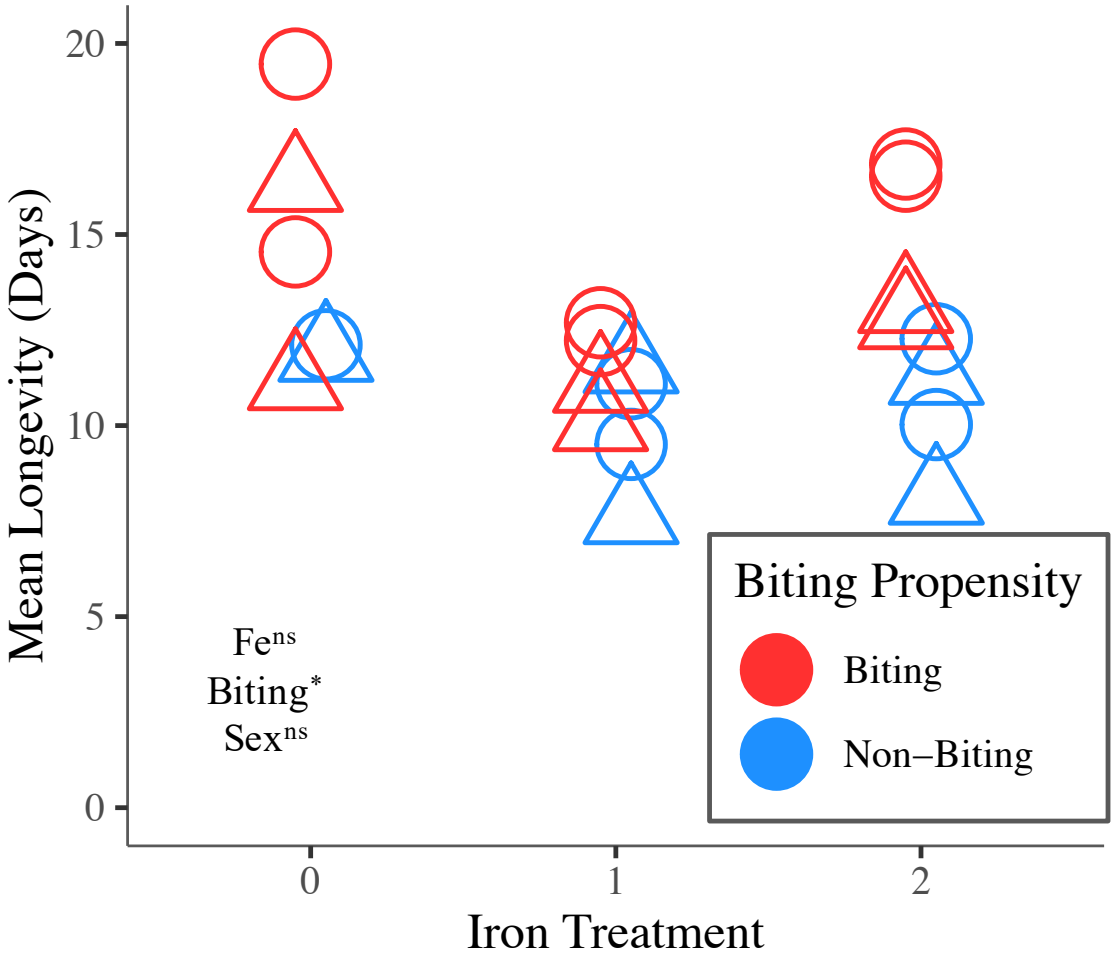

Figure 3: Adult longevity of males and females. Ferrous sulfate concentrations: 0 = 0mg/mL, 1 = 13mg/mL, 2 = 130 mg/mL. Biting propensities are offset by 0.2 units in width for visual comparison. Male longevity is indicated by triangles, female longevity by circles. ME0 male and female longevities were raised and lowered by 0.1, respectively, before plotting to avoid complete overlap of the data points. Significance of nested ANOVA (Appendix I) is given by: *P<0.05; ns, P ≥ 0.05).

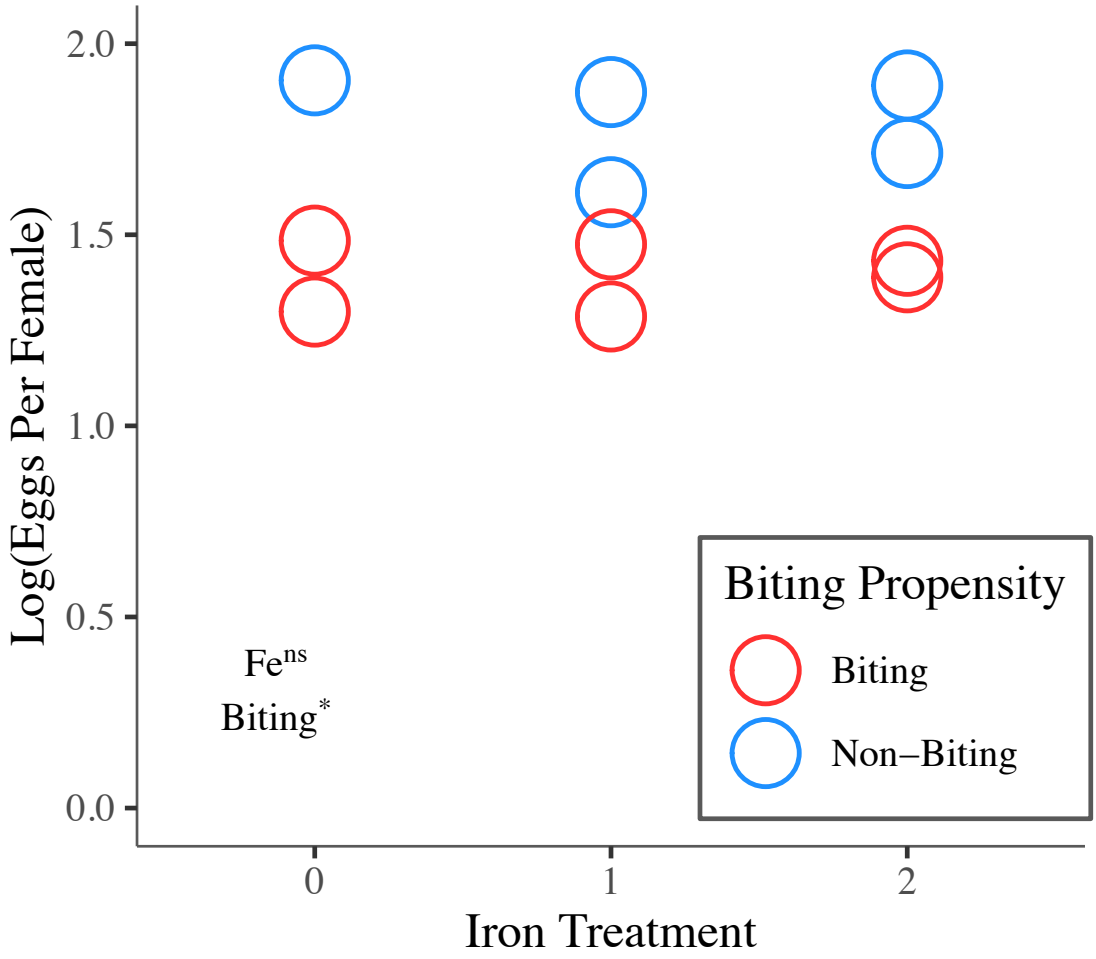

Figure 4: Lifetime fecundity (eggs per eclosed female). Ferrous sulfate concentrations as in Figure 3. Significance of nested ANOVA (Appendix I) is given by: *P<0.05; ns, P ≥ 0.05).

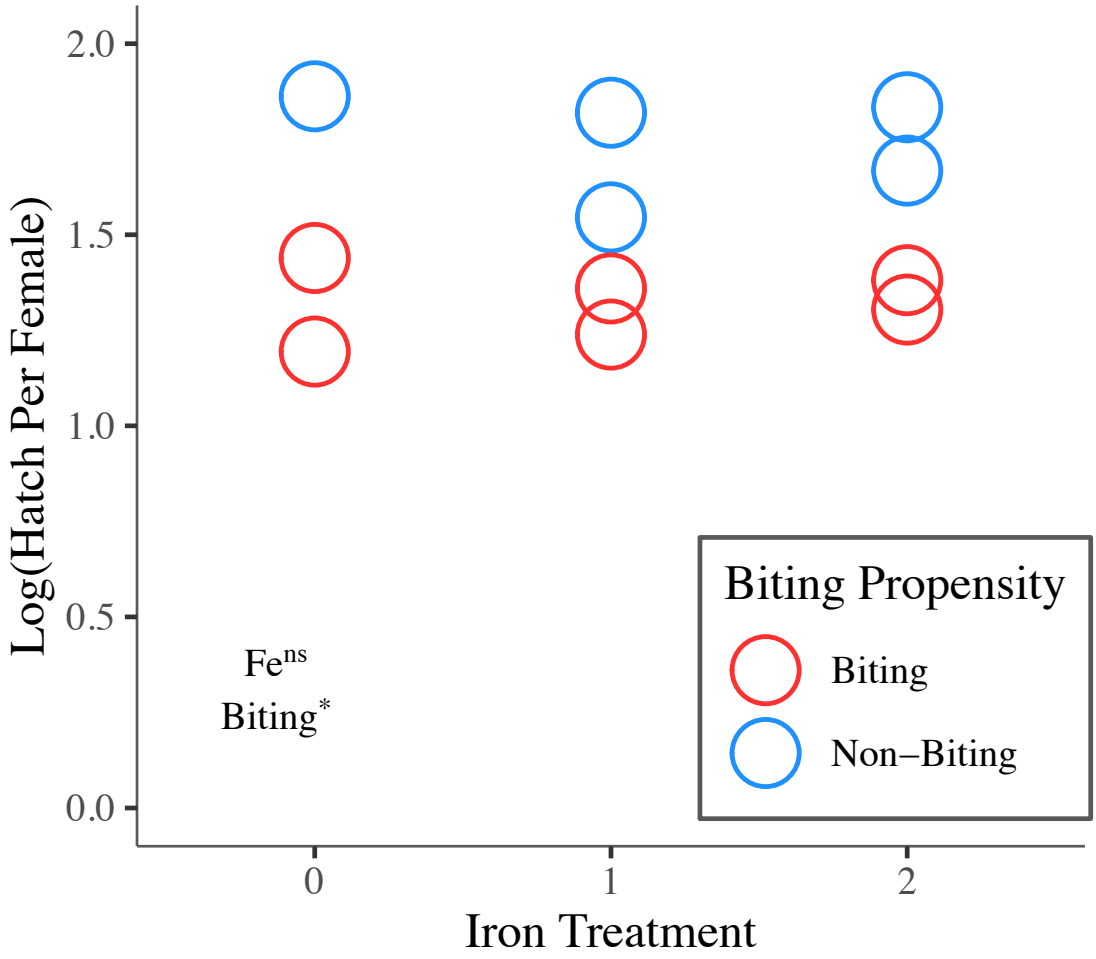

Figure 5: Lifetime reproductive success (number of hatched offspring per eclosed female). Symbols as in figure 4.

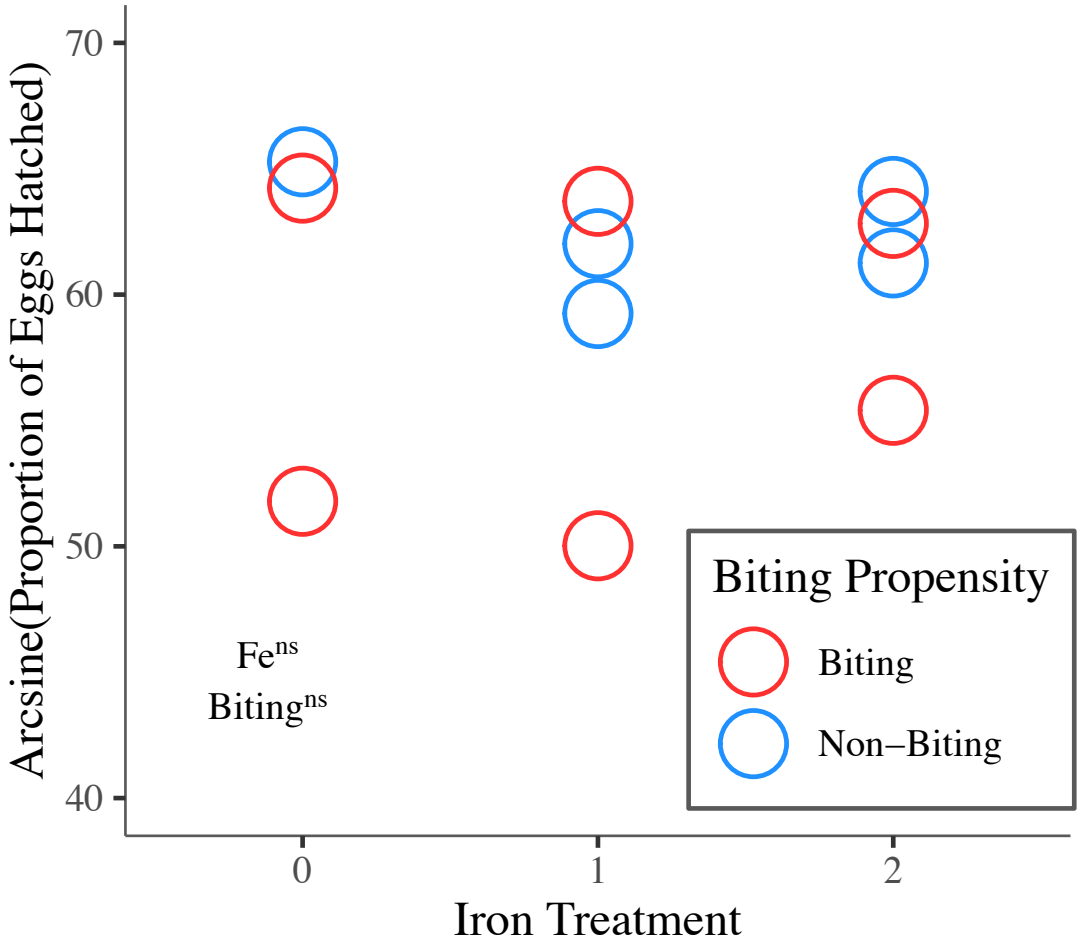

Figure 6: Fertility (frequency of eggs hatched). Symbols as in figure 4.

Discussion

My results show that non-biting populations of *Wyeomyia smithii* have higher fecundity and net reproductive success than biting populations, while biting populations experience greater longevity than non-biting populations. However, even concentrations of iron toxic to larvae did not significantly impact adult fecundity, fertility, longevity, or net reproductive success.

Previous research found increasingly deleterious effects on larval survivorship and adult emergence for *Wyeomyia smithii* exposed to ferrous sulfate solutions ranging from 0.003 mg/L to 33.583 mg/L (Rasmussen 2019). In contrast to larvae, iron, even at concentrations of ferrous sulfate approaching saturation levels and toxic to larvae did not affect adult longevity or reproduction in *W. smithii*, regardless of the biting propensity in their respective adult populations. Interestingly larval iron tolerance was greater in potentially biting than non-biting populations (Rasmussen 2019). In *Aedes aegypti*, other iron sources have shown larvicidal, pupicidal, and adulticidal effects (Ashokan et al. 2017). Although my findings do not support the use of ferrous sulfate as an environmentally safe means of vector control, other iron salts or other metal ions may still provide a promising method for controlling disease transmission by vector populations.

Appendices

Appendix I: Collated Data

Longevity:

Population	Treatment	Biting Propensity	Longevity
ME0 male	0	Non-Biting	11.981
ME0 female	0	Non-Biting	12.013
ME1 male	1	Non-Biting	11.577
ME1 female	1	Non-Biting	11.096
ME2 male	2	Non-Biting	11.277
ME2 female	2	Non-Biting	12.272
WI1 male	1	Non-Biting	7.630
WI1 female	1	Non-Biting	9.507
WI2 male	2	Non-Biting	8.142
WI2 female	2	Non-Biting	10.026
FL0 male	0	Biting	16.327
FL0 female	0	Biting	19.460
FL1 male	1	Biting	11.070
FL1 female	1	Biting	12.223
FL2 male	2	Biting	12.733
FL2 female	2	Biting	16.845
AL0 male	0	Biting	11.137
AL0 female	0	Biting	14.545
AL1 male	1	Biting	10.064
AL1 female	1	Biting	12.691
AL2 male	2	Biting	13.156
AL2 female	2	Biting	16.526

Fecundity:

Population	Eggs per Female	Treatment	Biting Propensity
ME0	80.188	0	Non-Biting
ME1	74.704	1	Non-Biting
ME2	77.754	2	Non-Biting
WI1	40.863	1	Non-Biting
WI2	51.743	2	Non-Biting
FL0	30.527	0	Biting
FL1	19.330	1	Biting
FL2	27.034	2	Biting
AL0	19.908	0	Biting
AL1	29.860	1	Biting
AL2	24.473	2	Biting

Net Reproductive Success:

Population	Hatch Per Female	Treatment	Biting Propensity
ME0	72.838	0	Non-Biting
ME1	65.974	1	Non-Biting
ME2	68.175	2	Non-Biting
WI1	35.118	1	Non-Biting
WI2	46.545	2	Non-Biting
FL0	27.491	0	Biting
FL1	17.330	1	Biting
FL2	24.050	2	Biting
AL0	15.643	0	Biting
AL1	22.882	1	Biting
AL2	20.145	2	Biting

Arcsine Percent Eggs Hatched:

Population	Arcsine (% Eggs Hatched)	Treatment	Biting Propensity
ME0	1.139	0	non-biting
ME1	1.083	1	non-biting
ME2	1.069	2	non-biting
WI1	1.034	1	non-biting
WI2	1.119	2	non-biting
FL0	1.121	0	biting
FL1	1.112	1	biting
FL2	1.097	2	biting
AL0	0.904	0	biting
AL1	0.873	1	biting
AL2	0.967	2	biting

Appendix II: ANOVA Results

Longevity

	Df	Sum Sq	Mean Sq	F	P
Treatment	2	43.03	21.517	1.104	0.369
Treatment: Bite Propensity	3	58.47	19.491	3.793	0.047
Treatment: Bite Propensity: Sex	6	30.83	5.138	1.201	0.380
Residuals	10	42.79	4.279		
Totals	21	175.12	50.425		

Arcsine Proportion Eggs Hatched

	Df	Sum Sq	Mean Sq	F	P
Treatment	2	0.00306	0.00153	0.242	0.794
Treatment: Bite Propensity	3	0.0189	0.00631	0.501	0.698
Residuals	5	0.0629	0.0126		
Totals	10	0.08487	0.020412		

Log(Eggs Per Female)

	Df	Sum Sq	Mean Sq	F	P
Treatment	2	0.0265	0.0133	0.0164	0.984
Treatment: Bite Propensity	3	2.436	0.812	8.902	0.0189
Residuals	5	0.456	0.0912		
Totals	10	2.918	0.9164		

Log(Hatch Per Female)

	Df	Sum Sq	Mean Sq	F	P
Treatment	2	0.0071	0.00357	0.0209	0.979
Treatment: Bite Propensity	3	0.512	0.171	9.329	0.0172
Residuals	5	0.092	0.01829		
Totals	10	0.6105	0.1925		

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