

ADULT LONGEVITY AND POTENTIAL TO TRANSMIT
DISEASE PATHOGENS IN THE PITCHER-PLANT
MOSQUITO WYEOMYIA SMITHII

by

ALLIE QUINN

A THESIS

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Title: Adult Longevity and Potential to Transmit Disease Pathogens in the Pitcher-Plant
Mosquito, *Wyeomyia smithii*

Approved: *Dr. William Bradshaw*
Primary Thesis Advisor

Approved: *Dr. Christina Holzapfel*
Primary Thesis Advisor

Mosquitoes have been called the deadliest animals on earth. Mosquito-borne pathogens kill millions of humans, livestock, and wildlife each year. Mosquitoes transmit disease through their saliva while taking a blood meal (biting). If there is no bite, there is no spread of disease, period. The pitcher-plant mosquito, *Wyeomyia smithii*, include blood-feeding southern populations that are fully interfertile with obligate non-biting northern populations. Selection on avid blood feeding in a southern population with both biting and non-biting phenotypes resulted in an increase in blood feeding from 20% to 80% in 19 generations. After eight more generations without access to blood, the incidence of biting reverted to 20%. However, it is not known whether biting occurs only once (one and done) or if females are capable of multiple biting episodes and what the reproductive consequences of multiple blood-fueled ovarian cycles are. I found that while *W. smithii* is capable of undergoing multiple ovarian cycles, propensity to bite did not change with ovarian cycle, and reproductive success from blood-feeding was actually reduced when compared with reproductive

success from a non-biting ovarian cycle. The unexpected reduction in reproductive success was due to a decline in number of eggs per batch and not a decline in embryonic viability. My results lead to the question of whether renewed selection on biting would result not only in an increased propensity to bite but also in an increased reproductive success through multiple blood-feeding ovarian cycles.

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Introduction

Mosquitos are the deadliest animals on Earth (CDC, 2018). In 2017, Malaria alone infected 219 million people and killed 435 thousand, with nearly half of the global population, especially children, at risk for contracting this disease. Many other deadly pathogens are spread by mosquitoes, such as Zika, dengue, yellow fever, and lymphatic filariasis, which combined kill millions more people yearly (WHO, 2019). Among mosquitoes, only the females bite and their threat lies in their ability to carry lethal viruses and then transmit them to a host through their saliva when biting. Consequently, if mosquitoes do not bite, they do not transmit diseases. Furthermore, the more times a mosquito bites in its lifetime, the greater is its ability to spread disease, its **vectorial capacity**¹. My thesis considers the ability to bite repeatedly and the reproductive consequences of multiple biting in the model mosquito, *Wyeomyia smithii*.

W. smithii lays its eggs and completes its pre-adult development only within the water-filled leaves of the carnivorous purple pitcher plant, *Sarracenia purpurea* (Smith, 1904; Bradshaw and Lounibos, 1977). The range of *W. smithii* extends from the Gulf Coast northwards into Canada (Merz et al., 2013) (Figure 1). All populations lay one batch of eggs without biting. Southern females require a blood meal from biting to support ovarian development and to produce a second batch of eggs; northern females never bite and yet are able to produce multiple batches of eggs (Bradshaw, 1980; O'Meara et al., 1981). Both southern and northern populations are fully interfertile (Bradshaw and Lounibos, 1977). Southern females are not universally biting, but

¹ **Vectorial capacity** is the ability of mosquitoes to acquire, carry, and transmit a pathogen. Among mosquitoes, only adult females bite and take a blood meal and transmit pathogens in their saliva while biting. Vectorial capacity increases with the number of bites a mosquito takes in her lifetime and the number of offspring she produces.

comprise 4-40% of the females in a population. Selection on biting in one southern population increased propensity to bite from 20% to 80% in 19 generations; hence biting and the production of nutrition for reproduction is a genetically-determined trait subject to selection. Biting in a southern population, or in the line selected for biting, results in increased lifetime offspring per adult female (Bradshaw, 1980, 1986; Bradshaw et al. 2018; Kirsch 2019). However, in these studies, all biting females were pooled in a single cage. It is not therefore known whether the increase in lifetime **reproductive success**² is due to a single biting-ovarian cycle or whether females will bite multiple times and undergo multiple, egg-producing ovarian cycles. Consequently, my research focuses on questions relating to the consequences of individual biting-ovarian cycles during the lifetime of females in a population previously selected for a high biting propensity. Specifically, I ask:

1. Do biting females take only one blood meal and then quit (“one and done”).

2. If biting females take more than one blood meal:

- 2A. Does propensity to take subsequent blood meals increase, decrease with sequential blood meals, or stay the same; i.e. are females that take their first blood meal more blood-thirsty, adversely affected (implying a cost of reproduction), or unaffected?

- 2B. What are the reproductive consequences of multiple biting episodes; i.e., how are **fecundity**³, **fertility**, and lifetime reproductive success (fecundity X fertility) affected by blood-based ovarian cycles?

² Number of live offspring produced per adult female emerging into the population

³ **Fecundity** is the number of eggs produced per female emerging into the population. **Fertility** is the proportion of those eggs that hatch.

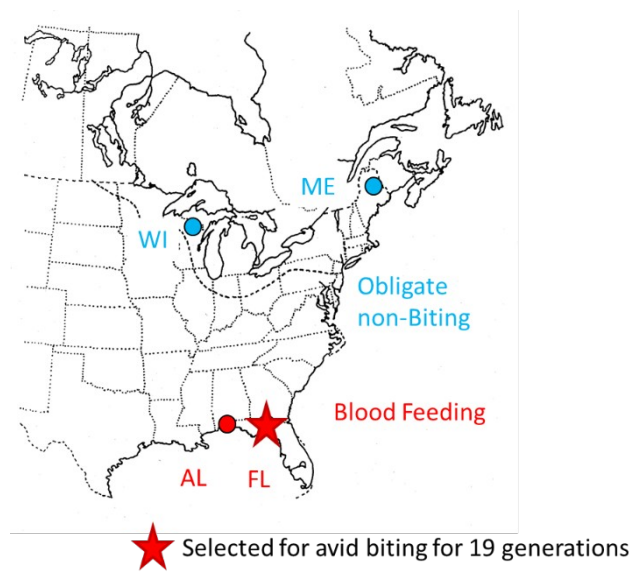


Figure 1. Map showing origins of several *Wyeomyia smithii* populations.

Blue indicates the origins of northern, non-biting populations, red indicates the origins of southern, biting populations, and the red star indicates the population selected for biting (WIOB) which was used in my experiment.

Broader Implications of my Research

Though *W. smithii* is not known to carry diseases that infect humans, they are part of a genus that does (Scherer et al., 1971; Srihongse and Johnson, 1965; Srihongse and Johnson, 1967). Specifically, the genus *Wyeomyia* is known to spread *Orthobunyavirus* (Chowdhary et al., 2012), a viral genus infecting mammals, birds, and even plants (Junglen, 2016). While many species in the *Wyeomyia* genus are tropical (Frank and Fish, 2008), as climate change intensifies it is likely that these tropical species will move north into the United States and other highly populated areas (Chowdhary et al., 2012). The basis for this prediction follows from models of *Aedes aegypti*. It is anticipated that the changing climate will drive this species north, bringing along dengue fever, chikungunya, Zika virus, and other diseases to the United States for the first time (Ryan et al., 2019). Consequently, if a similar response arises for the genus *Wyeomyia*, it is important to predict how these mosquitoes might spread disease in the temperate zone by determining the prevalence and consequences of blood feeding in their less-threatening congener, *W. smithii*. My research serves as a point of departure for these future enquiries.

Materials and Methods

Rearing Procedure

Approximately 1890 mosquitoes in the larval stage were taken from the 26th generation of the population know as Wilma offspring of biters (WIOB). This population was originally collected from Florida. Larvae were reared in larval medium consisting of 1.08×10^{-1} mg/mL tetracycline water and 2 mL of larval food. Larval food consisted of four parts Geisler Guinea Pig Feed, Sergeant's Pet Care Products and one part San Francisco Bay Brand freeze dried brine shrimp. Larvae and larval medium were held in 150 x 25 mm Petri dishes. Hibernating larvae were moved from the **short-day room**⁴ to the **long-day room**⁵ to induce development. Pupae were collected and separated into groups of 50 every second day. Pupae were placed in a transparent cup filled with deionized water. Three days after pupa collection, pupa cups were placed into a cage containing 6 raisins as a carbohydrate source and one *Sarracenia purpurea* leaf to stimulate oviposition.

Eggs were collected three times a week into a 150 x 25 mm Petri dish filled with deionized water. Eggs remained for 5 days in the short-day room. Hatch was then collected from egg dishes and placed 35-to-a-dish in 150 x 25 mm Petri dishes containing larval medium. To obtain the experimental populations, a selection of 840 individuals representing and proportional to the three highest hatch days were taken from the total hatch collected. These individuals were divided into three groups of 280

⁴ Short days promote and maintain hibernation; the **short-day room** is programmed for 0% humidity, a constant 21 °C, and 10 hours light per day.

⁵ Long days avert or terminate hibernation. The **long-day room** is programmed for > 80% humidity, a daily sine-wave temperature cycle oscillating from 15 °C to 32 °C each day, and 18 hours of light per day. The temperature cycle lagged the light cycle by 3h, as in nature.

larvae, then placed into the long-day room to induce pupation. Pupae were collected and placed in cages as before.

Blood Feeding Procedure

Because providing blood meals from a live source is time consuming and expensive, I employed Kirsch's (2019) blood feeding apparatus that uses commercially available sheep's blood (Figure 2). Due to logistical and time constraints, I performed my experiments on a single, southern *W. smithii* population called WIOB. Selection on biting had been applied to this population over the course of 19 generations, and then allowed to progress without biting for the past 8 generations resulting in a 19% incidence of biting (Kirsch, 2019).

Experimental animals were provided blood three times a week on a Tuesday, Thursday, Saturday schedule. They were offered blood during a consistent two-hour window from 12 to 14 hours after lights turned on in the long-day room to control for daily fluctuations in activity. 5 mL of defibrinated sheep's blood was heated to 45 °C in a water bath. The 20 mL syringe of the blood feeding apparatus was filled to the 5 mL mark with dry ice. Once the blood reached 45 °C, a cotton pledget was secured to the needle of the apparatus. The pledget was then dipped 1 cm deep into the warmed blood. The remaining blood was poured over the top end of the pledget. Water from the water bath was poured into the cup of the apparatus. The pledget part of the apparatus was then placed through a hole in the top of a cage and left for 20 minutes. Mosquitoes were deemed to have blood fed if blood was visible in their abdomen. Once a fed mosquito had freed its proboscis from the pledget, it was removed via aspirator and placed in its

respective new cage. Blood offerings ceased once two weeks had passed during which no mosquitoes took a blood meal.

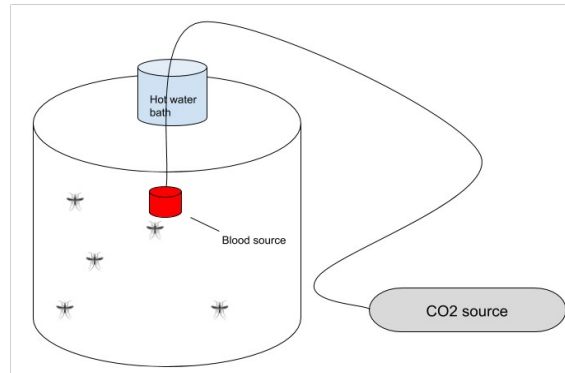


Figure 2. Kirsch's (2019) artificial blood feeding apparatus.

Heated CO₂ flowed into and out of a blood-soaked pledget to attract mosquitoes to the “host.”

Serial Transfer Method

For each replicate, blood was first provided to “Cage 1” (a cage containing mosquitoes that had not bit yet). Mosquitoes that took blood were moved via aspirator into a second cage, labeled “Cage 2” (which contained mosquitoes that had bitten one time). Blood was then offered to mosquitoes in “Cage 2.” Mosquitoes that took blood were moved into a third cage labeled “Cage 3” (which contained mosquitoes that had bitten two times). This process was repeated until all cages had been fed. The number of biters from each cage was recorded after each blood feeding session. Eggs were collected and counted from each cage on a Tuesday, Thursday, Saturday schedule. Eggs were placed into the short-day room where they sat for 5 days before hatch was counted. After first count, hatch dishes were checked every second day for additional hatch. Hatch dishes were dumped the first day that no new hatch was present. All cages

were equipped with 6 raisins as a carbohydrate source and one cut *Sarracenia purpurea* leaf to stimulate oviposition.

Calculations and Analysis

To analyze the biting propensity data, a χ^2_c incorporating Yates correction for continuity was used to determine if propensity to take a second (or more) blood meal was independent of the propensity to take the first bite. The p-value was also calculated to determine if the frequency of one-time biters was significantly different from the frequency of mosquitoes that took multiple bites. Propensity to bite two and three times was combined into one category due to the small sample size of three-time biters (violating assumptions of χ^2 analysis). An arcsine transformation was performed on the fertility data to approximate a normal distribution. Single-factor ANOVA's were performed on net-reproductive success, arcsine transformed fertility, and fecundity data. The variances obtained from these ANOVA's were used to calculate ± 2 standard errors, which were used for error bars on the graphs for these three data sets. Two-sample t-tests assuming unequal variances were performed on these same three data sets to determine if there were a significant difference between once-biters and twice-biters. Results were deemed significant when $p < 0.05$. All analyses were performed in Excel. Summary statistics are shown as insets in the figures; full ANOVA's and χ^2 table are provided in the Appendix.

Results

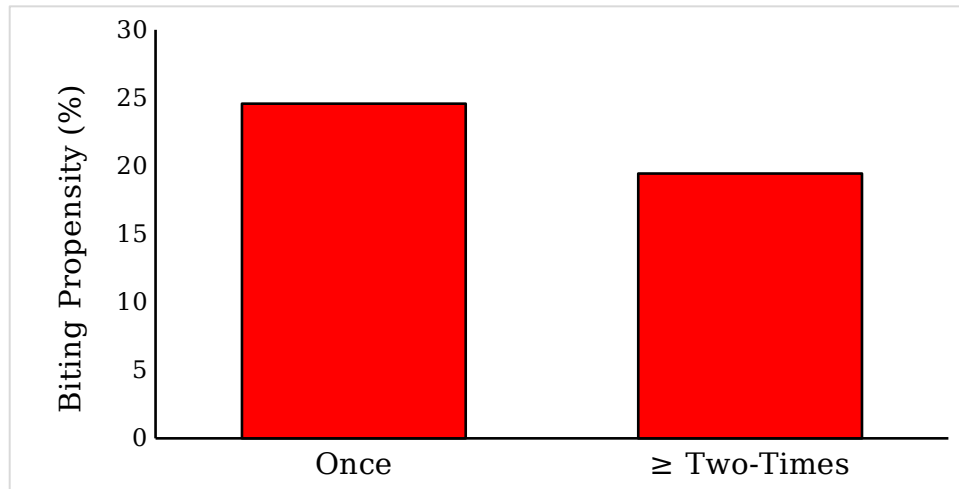


Figure 3. Propensity of mosquitoes to bite one time and two or more times.

My results show that *W. smithii* can take more than one blood meal (Figure 3). There was no significant change in the average propensity of mosquitoes to bite once (24.6%) and the average propensity of mosquitoes to take a subsequent bite(s) (19.4%).

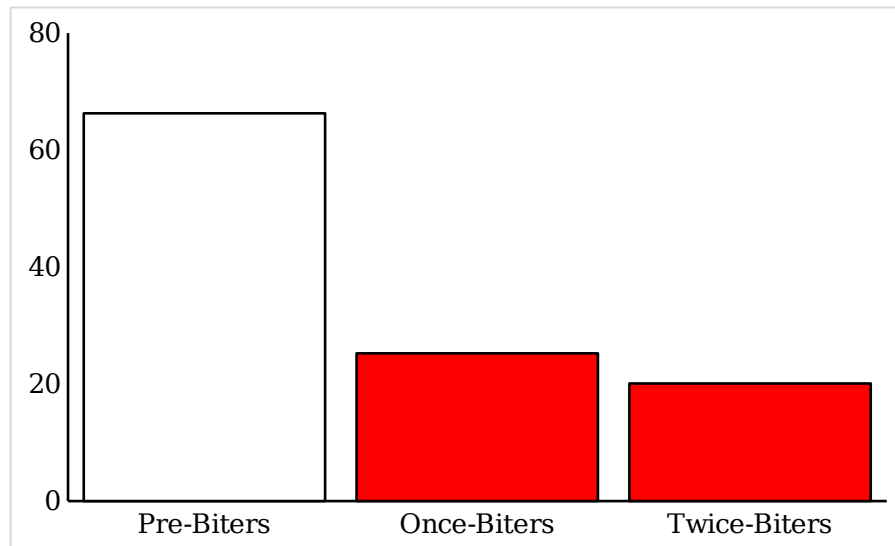


Figure 4. Net reproductive success measured in number of hatch per eclosed female for pre-biters, once-biters, and twice-biters.

Net reproductive success (Figure 4) of pre-biters (66.3 hatch per **eclosed**⁶ female) was significantly higher than net reproductive success of once-biters (25.2 hatch per eclosed female) and twice-biters (20.1 hatch per eclosed female). There was no significant difference in reproductive success between the second and third feeding/reproductive cycles.

⁶ **Eclosion** is the act of emergence of successive stages of development. Herein, eclosed females are those that have successfully emerged as flying adults from the aquatic pupal stage.

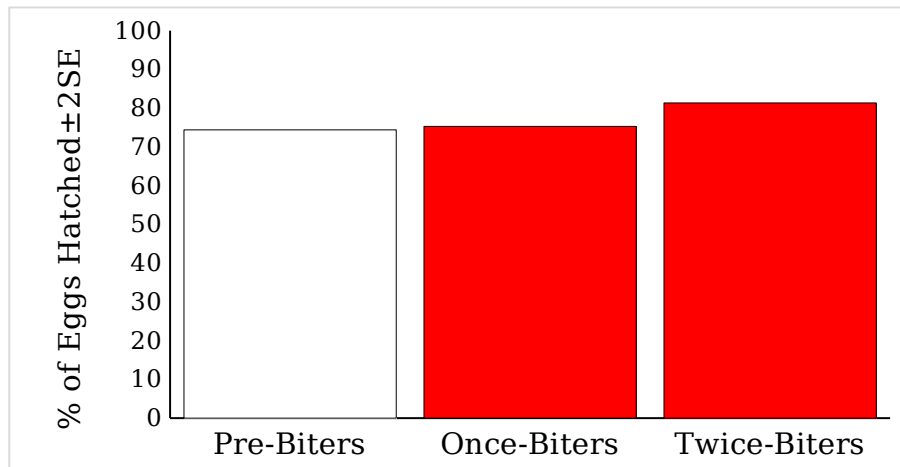


Figure 5. Arcsine transformed fertility measured in percent of eggs hatched.

To improve readability, radians have been converted to degrees for this graph.

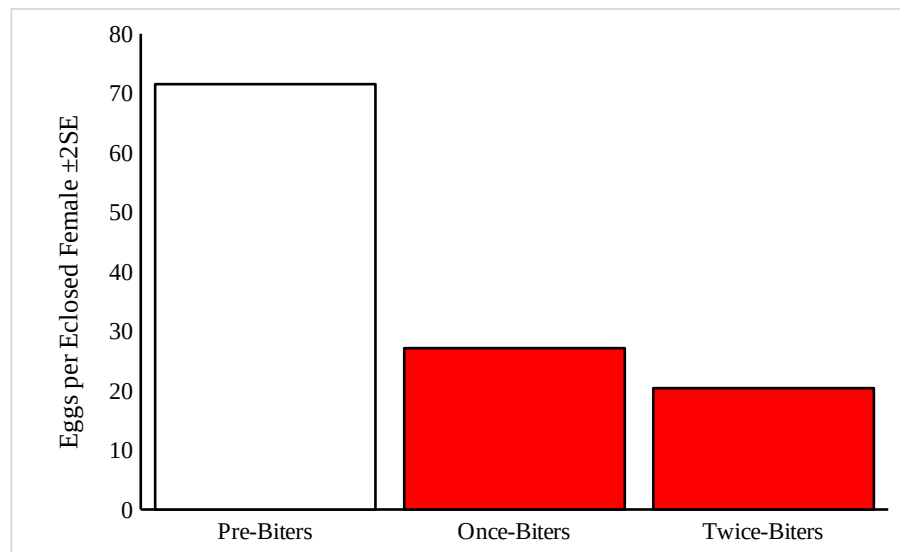


Figure 6. Fecundity measured in number of eggs per enclosed female for pre-biters, once-biters, and twice-biters.

Reproductive success is the product of fecundity and fertility. I found that there was no significant difference in fertility of pre-biters (1.298), once-biters (1.314), and twice-biters (1.420) (Figure 5), while fecundity decreases in biters as compared with

pre-biters. Fecundity of pre-biters (71.5 eggs per eclosed female) was significantly higher than fecundity of once-biters (27.1 eggs per eclosed female) and twice-biters (20.4 eggs per eclosed female). There was no significant difference in fecundity between once and twice biters (Figure 6).

Discussion

My results show that *W. smithii* females can bite more than one time, and are able to lay multiple clutches of eggs (Figure 3), thereby increasing their capacity to spread pathogens **horizontally** as well as **vertically**⁷. Biting propensity neither increases nor decreases showing that previous blood feeding neither enhances nor diminishes avidity for subsequent blood feeding.

Although propensity to bite remains the same for successive ovarian cycles, female reproductive success (hatch per eclosed female) is lower for blood-based as compared with the original non-blood feeding cycle (Figure 4). However, reproductive success does not decline with successive ovarian cycles (Figure 4) due to sustained fecundity (Figure 6) without a concomitant loss of fertility (Figure 5). Hence the decline in reproductive success from non-biting to biting cycles is not due either to female age or sperm depletion, but rather related to differences between larval-acquired resources and resources acquired through adult blood feeding.

One explanation for the decrease in reproductive success is the high extrinsic cost of biting. Specifically, the energetic investment required to seek out and take multiple bites and then metabolically convert that blood to nutrients available for ovarian maturation (Graça-Souza et al. 2006; Zhou et al. 2007; Benoit et al. 2011; Lahondère and Lazzari, 2012; Farjana and Tuno, 2013; Whiten et al. 2018; Bradshaw et al. 2018) may divert resources away from blood-feeding cycles.

⁷ In **horizontal** transmission, females must bite an infected host before they are able to transmit a pathogen (examples: malaria; dog heartworm). In **vertical** transmission, females acquire pathogens either as eggs from their mothers or in the seminal fluid from their mates; hence they can transmit pathogens at their very first bite, as well as subsequent bites (examples: LaCrosse, yellow fever, dengue, Zika, chikungunya & West Nile viruses).

Females achieving blood-derived, multiple ovarian cycles realize greater lifetime reproductive success compared to females who bite only once or not at all. Propensity to bite varies genetically both among and within populations of *W. smithii* (Bradshawsaw, 1980; Borowczak, 2017; Bradshaw et al., 2018). Selection on blood feeding increased propensity to bite from 20% to 80% (Borowczak, 2017) in 19 generations. Relaxing selection for eight generations resulted in the reversion of biting to the ancestral frequency of 20% (Kirsch, 2019). However, relaxation of selection may have left propensity of avid blood feeding and multiple biting cycles cloaked in the genetic background within the reverted biting line.

Such latent genetic variation would greatly enhance how fast renewed selection for blood-feeding could expend in populations during periods of climate change or other altered environmental conditions. Selection for blood feeding could be imposed in parallel on the ancestral, reluctant biting line and the reverted, selected biting line. Response to this selection should reveal whether enhanced genetic propensity to blood feed can reside cryptically in extant or invasive populations, enhancing how fast mosquitoes could evolve avid blood feeding in response to natural selection.

Going forward, my research has established multiple blood-fed ovarian cycles in a single line of *W. smithii* previously selected for avid biting. However, my results leave open the question of whether selection on blood feeding also results in increased blood-fueled ovarian cycles, i.e., a positive genetic correlation between propensity to bite and multiple biting episodes.

If such a correlation exists in *W. smithii*, it would lead to asking the same question in other mosquitoes, including those transmitting heinous pathogens among

humans, livestock, and wildlife. Such a genetic correlation would greatly amplify the evolutionary rate of vectorial capacity of these other mosquitoes in response to selection due to altered environmental circumstances, including climate change and invasive species.

Appendix

Table 1. Single factor ANOVA for net reproductive success (hatch per eclosed female)

Source of Variation	SS	df	MS	F	P-value
	3847.7		1923.8	34.976	0.0004
Between Groups	32	2	66	6	93
	330.02		55.004		
Within Groups	63	6	38		
	4177.7				
Total	58	8			

Table 2. Single factor ANOVA for fertility (% of eggs hatched, arcsine transformed)

Source of Variation	SS	df	MS	F	P-value
	0.0262		0.0131	0.5361	0.6106
Between Groups	39	2	19	48	22
	0.1468		0.0244		
Within Groups	19	6	7		
	0.1730				
Total	58	8			

Table 3. Single factor ANOVA for fecundity (eggs per eclosed female)

Source of Variation	SS	df	MS	F	P-value
	4631.1		2315.5	37.938	0.0003
Between Groups	24	2	62	74	94
	366.20		61.034		
Within Groups	54	6	24		
	4997.3				
Total	29	8			

Table 4. Chi-square calculator with Yates correlation

	Once	Twice or more	Marginal Row Totals
Bite	74 (71.58) [0.08]	14 (16.42) [0.36]	88
No Bite	301 (303.42) [0.02]	72 (69.58) [0.08]	373
Marginal Column Totals	375	86	461 (Grand Total)

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