

HOLOCENE LEGACY: EVOLUTION OF THERMAL TOLERANCE AND
BLOODFEEDING IN THE PITCHER-PLANT MOSQUITO,
WYEOMYIA SMITHII

by

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DISSERTATION ABSTRACT

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Title: Holocene Legacy: Evolution of Thermal Tolerance and Bloodfeeding in the Pitcher-Plant Mosquito, *Wyeomyia smithii*

The legacy of historical biogeography impacts many organisms and results in a wide range of character variation over a latitudinal gradient. The pitcher-plant mosquito *Wyeomyia smithii* is one such organism that demonstrates a wide range of phenotypic and genotypic variation over the entirety of its range from the Gulf Coast to Canada. A geographic cline established by the presence and recession of the Laurentide Ice Sheet is manifest in the narrow range of thermal tolerance exhibited by different populations and also in the differing propensity of bloodfeeding by these mosquitoes. These contemporary clines were analyzed by a variety of experimental methods ranging from year-long fitness assays, scanning electron microscopy, and RNA-sequencing to determine the patterns underlying the resulting evolutionary differences among established populations.

This dissertation includes both unpublished and co-authored material.

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To my parents, who gave me their love of adventure and discovery.

To Dan, with as much love as you have given me.

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CHAPTER I

INTRODUCTION: GEOGRAPHIC VARIATION IN NATURAL POPULATIONS OF THE MOSQUITO *WYEOMYIA SMITHII*

Latitudinal gradients can be observed in many abiotic environmental effects, such as in the variation of temperature, humidity, and photoperiod. Along both the east and west coasts of North America the effect of these gradients is seen through drastic temperature, humidity, and day length changes throughout the year. Northern areas tend to have cold winter temperatures and long summer days, while southern latitudes have hotter summers with less change in the day length between summer and winter. Any organism that has a range over these different environments would therefore have to be able to adapt to these varying conditions. The resulting phenotypes of these organisms mirror the effects of the environment, resulting in clines¹. These phenotypic clines can then be used to compare the effects of latitude among different populations of the same species.

The effects of recent climate change are closely tied into established latitudinal gradients. The rate of climate change has been noted to be progressing more rapidly within higher latitudes² and the predicted effects on organisms that have a wide latitudinal range are varied. In mid to high latitudes, the population growth rates of insects are projected to increase; however, within lower latitudes population growth rates are expected to significantly decrease³. These predictions are based on the observation that insects at lower latitudes exist very close to their lethal thermal limits and it is predicted that insects at higher latitudes have a wider range of thermal tolerance^{3,4}. Given

that rates of climate change are increasing in northern latitudes, I performed an experiment to determine the thermal tipping point in populations across a wide latitudinal range (30°N to 51°N) which is detailed in Chapter II.

The study of phenotypic differences among populations across a wide latitudinal gradient has an established historical basis, and numerous observations for traits in organisms ranging from trees to fish have been recorded⁵⁻⁸. Organisms within the same species can have different phenotypes depending on the specific selective processes within their local environments, and these differences can be significant⁹. The traits could be involved in very important life-historical events that impact an organism's chances for survival, which makes these traits interesting to study due to their vital nature^{9,10}. A mosquito's ability to bloodfeed is an example of such an important trait: a blood meal will provide the mosquito with nutrients to produce eggs, and its entire reproductive success may depend on that blood meal¹¹. Chapters III and IV will expand upon the significant differences in the bloodfeeding trait between populations of a single species of mosquito.

An organism that is especially well-suited for the study of biological variation across latitudes is the mosquito *Wyeomyia smithii*. *Wyeomyia smithii* is a small mosquito that closely follows the range of its host plant, the carnivorous pitcher plant *Sarracenia purpurea*, along the east coast of North America from as far south as the Gulf Coast of Florida to the far reaches of Quebec and Newfoundland. Due to the stationary nature and the long generation times of their host plant, populations of *W. smithii* are well-established and can be sampled continuously over many years. In this dissertation, I will examine the evolution of thermal tolerance and the patterns of bloodfeeding seen within

the populations of a single species of this mosquito. Southern populations (below 39°N) have always been southern, but northern populations are much more recently derived from populations that survived along the edge of the Laurentide ice sheet, which disappeared around 20,000 years ago within the Holocene epoch^{12,13}. Over the past 20,000 years leading up to the present, gradual and rapid climate warming and range expansion have all contributed to gradients of thermal tolerance and bloodfeeding propensity within *W. smithii*. Herein, I show that we can use the legacy of latitudinal variation generated within the Holocene epoch to examine both the physical and genetic effects of such variation. I can then incorporate those results into an evolutionary framework of *W. smithii* and into the broader aspects of biological variation over geography.

Within Chapter II, I will investigate the concept of a thermal tipping point in temperate populations leading to either persistence or extinction. Many organisms require a certain thermal minimum to complete general biological processes, and there is an optimal temperature for these organisms to exist. However, it has been shown that the optimum temperature for survival is very close to lethal temperatures, resulting in a sharp drop in survivorship if that thermal limit is exceeded^{4,14}. Organisms therefore exist very close to their lethal limits for survival to maintain optimum efficiency. Many of the thermal tipping point studies, however, have been limited in scope because they either raise organisms in constant temperatures or for short periods of time, both of which are not natural conditions for any organism. Herein, I show the first study that raises organisms in a simulated natural environment that incorporates both a daily and seasonal temperature variation and integrates the fitness of these organisms over the course of an

entire year, also known as the yearlong cohort replacement rate¹⁵. This chapter will be published as a research article, and the co-authors for the chapter are Dr. William Bradshaw and Dr. Christina Holzapel.

Chapter III introduces my investigations into the evolutionary loss of bloodfeeding in northern populations of *W. smithii*. As *W. smithii* expanded its range beyond the Gulf Coast and North Carolina coastal plains, the ability to bloodfeed was lost in more northern populations. Older populations in the south still retain the ability to bloodfeed, but younger populations in northern areas (above 36°C latitude) exhibit a complete lack of this character, which is unique within a single mosquito species^{11,16}. We therefore have a powerful system in which to examine the evolutionary loss of bloodfeeding for eventual application to vector biology and disease transmission. Mosquitoes are one of the most important vectors for human disease transmission and are responsible for thousands of deaths annually throughout the world^{17,18}, and research that investigates the reasons for a loss of biting in natural populations could help with vector control.

The first investigation concerns any morphological differences in the mouthparts between northern and southern mosquitoes that could be a reason for the loss of bloodfeeding. Within this chapter, scanning electron micrographs are used to analyze the maxillae and maxillary teeth of both biting and non-biting mosquitoes. The study will be published as a research article, and the co-author for this chapter is Rudy Borowczak. R.B. assayed the propensity to bite for all of the populations, while I collected the microscopy samples, performed the imaging, and performed the analysis. Both R.B. and I wrote the paper.

Chapter IV explores the concept of the evolutionary loss of bloodfeeding at the molecular level. Within this chapter, I describe an RNA-sequencing study performed to determine if there are 1) differential expression profiles of mRNA transcripts between biting and non-biting populations, and 2) if these transcripts have a putative function that could be correlated with the loss of biting in northern populations. RNA-sequencing is a new method that has recently come out of the advent of next-generation sequencing technologies (NGS)¹⁹. Messenger RNA is isolated from the tissue/organism of interest and reverse-transcribed to cDNA, which is then sequenced and assembled to produce a transcriptome. These technologies generate millions of short-read sequences (35-500 base pairs) with enormous sequencing depth that can then be assembled into genomes or transcriptomes and used for comparative studies^{20,21}. Transcriptomes are much smaller than genomes and therefore can be assembled relatively quickly into contiguous sequences (contigs) that provide a representation of all the coding genes in the tissue or organism at the time of sampling^{21,22}.

This study is once again made more powerful by the unique situation presented by *W. smithii*, in which we can study the evolutionary loss of biting within a single interfertile species. Although *W. smithii* is not a historically established model organism and lacks a fully sequenced genome, technological advances within the past decade have provided a reliable method for comparative genomics and transcriptomics of historically non-model organisms^{19,22,23}. Even without a reference genome, transcriptome assembly has been proven to be a reliable tool for population genomic and differential expression analyses for a number of organisms²⁴⁻²⁸.

The RNA-sequencing within this chapter is presented as a comparative study between a biting population, a non-biting population, and a population selected for increased biting. These comparisons allow us to identify genes that are correlated with the act of biting and with the evolutionary loss of biting. This study will be published as a co-authored research article. Dr. William Bradshaw, Dr. Christina Holzapfel, Dr. William Cresko, and I conceived of this experiment. I collected the samples, performed the experiments, and ran the analyses. Dr. Bradshaw, Dr. Holzapfel, Dr. Cresko, and I wrote the paper.

The following dissertation explores the differences in evolutionary history brought about by the receding glaciers and the resulting latitudinal clines within the Holocene epoch in both thermal tolerance and bloodfeeding propensity in a single species of mosquito. These studies illustrate the importance of integrating evolutionary history with geographic variation to answer a wide range of important biological questions for the mosquito *Wyeomyia smithii* and other organisms.

CHAPTER II

ON A RAZOR'S EDGE: 1°C TIPPING POINT BETWEEN SURVIVAL AND EXTINCTION

William E. Bradshaw and Christina M. Holzapfel conceived of the study and designed the experiments; Alida T. Gerritsen ran the experiments; W.E.B., C.M.H. and A.T.G. analyzed the data and wrote the paper.

Introduction

“Tipping points” refer to small changes in the environment that cause an abrupt and usually irreversible crossing of a large-scale climatic threshold^{1,2}. The concept of a tipping point can also refer to plant and animal populations where a small change in the environment can lead to local or regional extinction. This concept has usually been applied to response by tropical organisms to climate warming because they already are thermal specialists living close to their maximum thermal tolerance^{3,4}. Herein, we apply this concept to temperate populations. Unlike previous studies, we rear experimental populations of mosquitoes in their natural microhabitat and assess performance through all four seasons in a natural thermal year. We show that during hot summers the difference between population persistence and extinction can be due to as little as a 1°C shift in the maximum temperature on the hottest day of the year and that latent effects of a hot summer can persist through winter and be manifest in a loss of fecundity the following spring. Furthermore, short-term survival and reproduction at sub-threshold temperatures do not necessarily provide a reliable indicator of population vulnerability to

thermal stress under natural conditions. What appear to be viable populations can face extinction with only a small increase in temperature during only a brief period of their entire thermal year.

Common measures of organismal performance in different thermal environments include locomotion, feeding/assimilation, growth, development, reproduction, recovery time after acute thermal stress, and survival⁵. All of these measures can affect fitness but all except survival of individuals or extinction of populations are potentially reversible. Herein, we consider reproduction, survival, and extinction of populations when confronted with hot summer weather followed by a typical ensuing winter and spring.

The mosquito *Wyeomyia smithii* completes its preadult development only within the water-filled leaves of the purple pitcher plant, *Sarracenia purpurea*. The range of the mosquito follows that of its host plant from the Gulf of Mexico to northern Canada^{6,7}. By running experiments with the mosquito reared in the leaves of intact plants, we are able to assess fitness in the mosquito's natural microhabitat, thereby mitigating the effects of a novel environment⁸⁻¹⁰. Like most temperate plants and animals¹¹⁻¹³, *W. smithii* is photoperiodic, using day length to initiate, maintain, and terminate a larval diapause throughout its range⁶. While transplants between altitudes¹⁴⁻¹⁸ or longitudes¹⁹ can isolate the effects of temperature or moisture, transplants between latitudes²⁰⁻²², are always biased by differences in day length between latitudes²³. We agree that any reciprocal transplant experiments are bone fide tests for local adaptation but not necessarily for the

specific causality of local adaptation being due to temperature unless the confounding factors of day length and humidity are factored out of the experimental design.¹

With *W. smithii*, we are able to run latitudinal transplants in unique computer-controlled rooms where we can create any climate from the tropics to the arctic by programming field-based annual temperatures, day lengths and humidity independently. We are therefore able to factor out genetic differences in seasonal response among populations due to day length and humidity (Fig. 1), thereby allowing us accurately to assess temperature as an isolated variable.

To test for thermal tolerance among 12 populations over a latitudinal gradient from the Gulf of Mexico to Newfoundland (30-50°N), we exposed mosquitoes in the leaves of intact pitcher plants to a southern thermal year where the maximum daily temperature on the hottest day of the year was 39°C (Fig. 2a). Under these conditions, weekly production of offspring declined with increasing temperature until only two of the original 12 cohorts persisted after the peak summer temperature of 39°C (Fig. 2b). Both of the remaining populations were from the Gulf Coast and went through two subsequent summer generations, after which we concluded that the two remaining populations were viable and terminated the experiment.

¹ In this paper, we refer to adaptation in its evolutionary sense: An adaptation is an evolutionary (genetic) response to selection by a population that confers increased fitness or performance in the selected environment as compared with the ancestral environment.

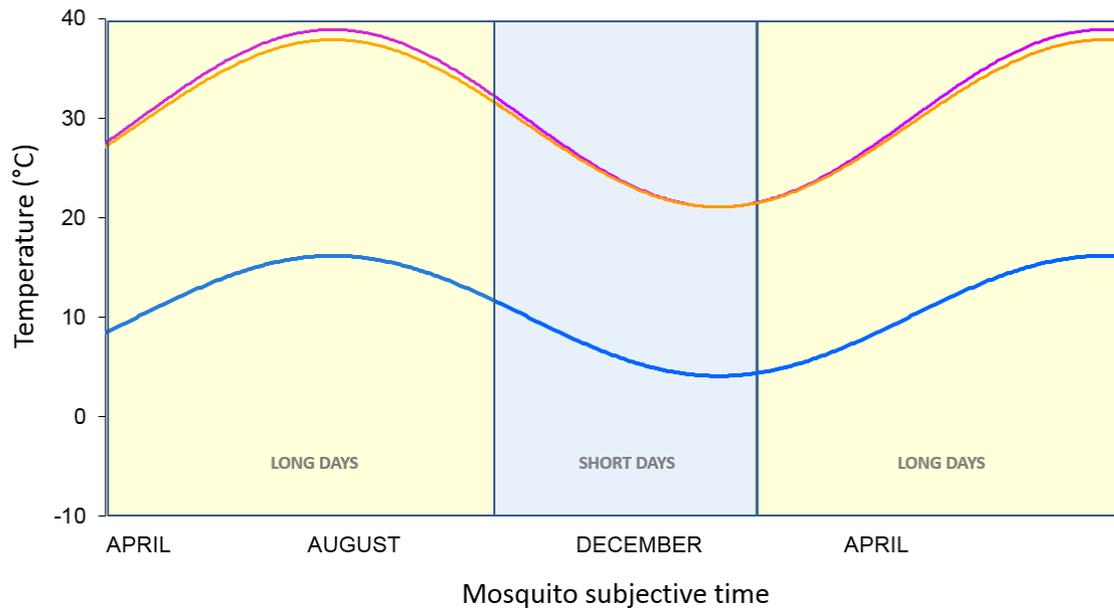


Figure 1. Southern climate. Daily temperatures oscillated with a sine function between a daily maximum (purple & orange curves) and minimum (blue curve); likewise, the daily maxima and minima oscillated with a sine function between the annual maximum and minimum. Day lengths were either long (Light:Dark = 16:8) or short (L:D = 10:14) providing unambiguous long days promoting continuous development or short days promoting the onset and maintenance of larval diapause for all populations at all latitudes. Two thermal years are illustrated, one with the maximum daily temperature on the hottest day of the year set at 39°C (purple curve), the other at 38°C (orange curve). The initial cohorts of freshly hatched larvae were introduced into the leaves of intact pitcher plants starting on April 1st at a rate of 60 larvae per week for four weeks. Cohorts of 20 or 30 freshly hatched larvae from succeeding generations were introduced into leaves, up to a maximum total of 60 larvae per week, until hatch declined to zero as a consequence of short-day induced larval diapause in the fall. Larval censuses were made in the winter and two months later in the early spring.

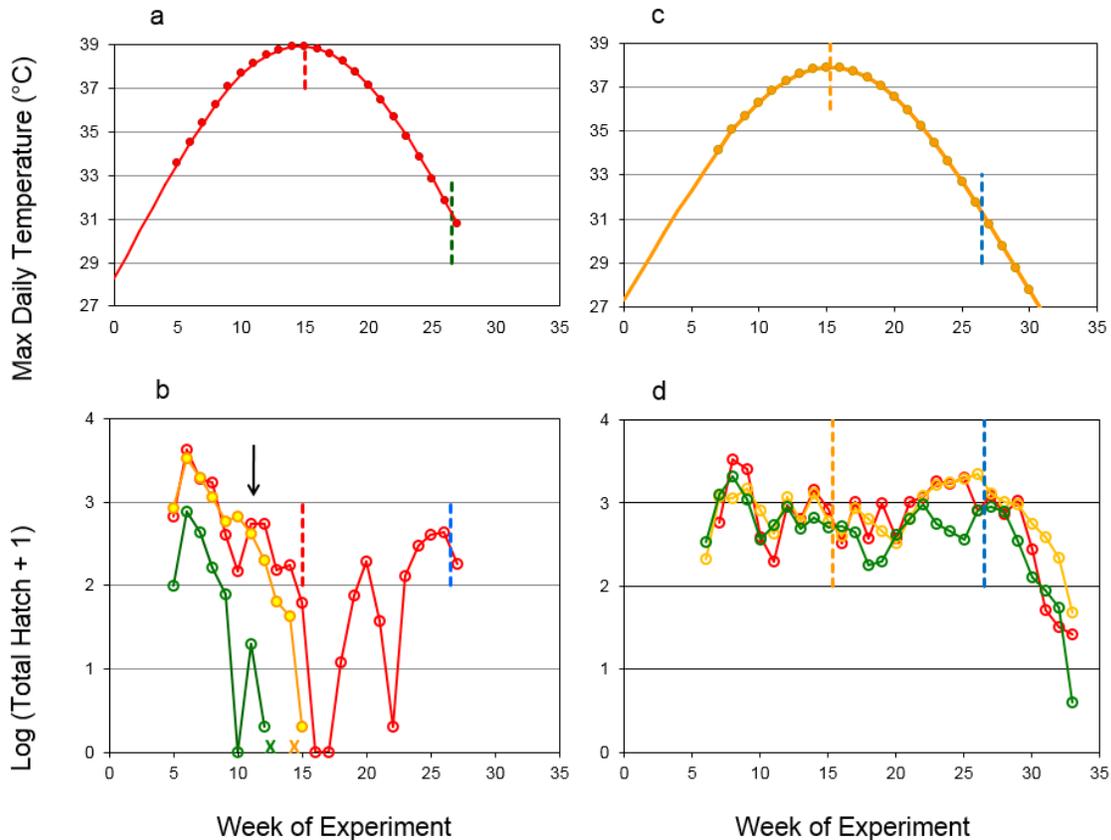


Figure 2. Thermal profiles and mosquito reproduction during the spring, summer and fall. a, c. Maximum daily temperatures. The daily maximum on the hottest day of the year is indicated by the red (39°C, a) or orange (38°C, c) vertical dashed line. Recordings of temperature on the hottest day of the year are shown in Extended Data Figure 1. The experiment was started on week 0 (April 1). Dots on the line show maximum daily temperature for each week of the experiment that larval hatch occurred. The dashed vertical blue line indicates the switch from long to short days. b. Total hatch per week for the southern (red), mid-latitude (orange) and northern (green) regions when the maximum daily temperature on the hottest day of the year set at 39°C. The northern populations all went extinct by week 12 (green X); the mid-latitude populations all went extinct by week 16 (orange X); the southern populations did not all go extinct. Rather, they likely persisted through the heat of the summer as heat-tolerant eggs and larvae in the pitcher-plant leaves³¹. The black arrow indicates the week with a maximum daily temperature of 38°C. Red and blue dashed lines as in a and c. d. As in b, except the maximum daily temperature on the hottest day of the year set at 38°C.

Given that widespread extinction occurred when the maximum daily temperature was set at 39°C, the question then became what would constitute a non-lethal maximum daily temperature? We noted that in the initial experiment, viable offspring were still being produced by all regions at 38°C (Fig. 2b, black arrow). Consequently, we re-programmed the room for a maximum daily temperature on the hottest day to be 38°C (Fig. 2c).

With a maximum daily temperature of 38°C, all but one of the populations reproduced continually until the onset of short days, entered larval diapause, and survived until the following spring (Table 1). Offspring production continued throughout the summer and fall until the imposition of short days (Fig. 2c,d) induced the normal process of larval diapause, which prevents metamorphosis to adults and subsequent reproduction. The number of larvae in diapause during the winter and surviving until the onset of long days in the spring did not differ among the three geographic regions (Table 1). However, the number of mosquitoes actually emerging as adults, the number of emerging adult females, and the number of vernal hatch declined with increasing latitude of origin (Table 1, from south to north). Eggs per eclosed adult female did not differ among latitudes of origin so that variation in vernal offspring production among regions was due primarily to survivorship of post-diapause larvae and pupae to adult eclosion and not subsequent female fecundity. Using the year-long replacement rate as an integrated index of fitness, there was no significant difference in fitness among regions (Kruskal-Wallis test: $\chi^2 = 3.96$, $df=2$, $P = 0.14$) in the 38°C thermal year.

Region	Pop ¹	Number of larvae		Adults	Females	Eggs per	Vernal	Ry ²
		Winter	Spring	Eclosing	Eclosing	Female	Hatch	
North	GM	37	30	30	13	26.2	250	1.04
North	DL	206	167	46	19	8.5	131	0.55
North	KC	81	81	25	8	7.5	33	0.14
North	ML	141	127	0	0	0.0	0	0.00
Mid-Lat	HV	236	218	72	25	17.0	425	1.77
Mid-Lat	MM	198	194	94	36	27.6	715	2.98
Mid-Lat	NP	185	185	75	31	12.2	308	1.28
Mid-Lat	PB	183	183	77	28	12.1	319	1.33
South	BC	166	166	136	60	18.2	1048	4.37
South	CI	0	0	0	0	0.0	0	0.00
South	LI	219	217	198	96	6.5	508	2.12
South	WI	207	174	174	82	19.8	1582	6.59
	χ^2	1.24	0.89	129.87	83.63	1.1	1050.89	3.96
	<i>P</i> (2 <i>df</i>)	0.054	0.064	6.30E-29	6.91E-19	0.1	6.35E-229	0.138

χ^2 tests are for independence of regional totals, except for Ry, which is evaluated with the Kruskal-Wallis Test.

¹Geographic details provided in Extended Data Table 1.

²RY = year-long per-capita replacement rate = (Vernal Hatch/240)

Table 1. Population censuses during the 38°C maximum thermal year.

The perception of fitness consequences of a hot summer during the 38°C thermal year depends upon the populations sampled. Southern populations achieved the highest (WI) and lowest (CI) year-long replacement rates (Table 1, RY); mid-latitude populations all replaced themselves (RY>1.0); northern populations achieved year-long replacement rates ranging from 0.00 to 1.42. Had we sampled only one population from each latitudinal zone, we could have concluded no thermal adaptation, co-gradient adaptation, or counter-gradient adaptation depending on the populations we sampled. Our results therefore underscore the importance of sampling multiple populations within each zone of putative thermal selection in tests of thermal adaptation based on natural populations.

At the level of individual measures of performance, not all of the effects of a hot summer environment were immediately apparent. Extinctions in the 39°C maximum thermal year were blatant; but, in the 38°C maximum thermal year, there was no differential survivorship through summer, fall, and winter among geographic regions (Table 1, Spring Larvae). However, subsequent adult eclosion, female eclosion, and, consequently, total offspring (hatch) produced in the spring declined from south to north. At the same time, the number of eggs produced per female did not vary among regions. These results show that there were latent effects of heat during the previous summer that differentially affected post-diapause survivorship and reproduction among latitudinal regions during the following spring. Even though not leading to extinction, or even an apparent immediate loss of fitness, thermal stress can lead to a loss of resilience, even after the population is returned to more benign environmental conditions²⁴⁻²⁵.

The precipitous tipping point is especially apparent at mid-latitude populations where a 1°C difference in maximum temperature on the hottest day of the year made the difference between all populations going extinct (Fig. 1b) and all populations achieving positive fitness (Table 1, $R_y > 1.0$). Even though a population may not currently experience or approach limiting temperatures on average, “the number of observed local monthly heat records around the globe is now more than three times as high as expected in a stationary climate”²⁶. Transient heat extremes for a few days can reduce the “thermal safety margin” of ectotherms²⁷. Temperate ectotherms usually experience body temperatures in excess of air temperatures and close to their limit of thermal tolerance²⁸. Survivorship of a mid-latitude population when confronted with a simulated four-day warm weather front declined from 60% in embryos to 40% in larvae to 5% in pupae and,

among surviving pupae, subsequent embryonic viability was reduced seven-fold compared to the absence of a warm weather front²⁹. Northern ectotherms exist at lower normal temperatures, but, like *W. smithii* (Table 1) they also may exist closer to their limit of thermal tolerance.

We conclude that thermal safety margins among temperate populations can be much narrower than is generally appreciated. Thermal tipping points among temperate populations can be very real, highly precipitous, and render populations subject to rapid extinction. Subtle changes in just 1°C in the maximum daily temperature over a brief period during the summer can determine the difference between persistence and extinction not only of individual populations, but also among all populations within an entire climatic region. The increased incidence of even transient heat waves only serves to exacerbate these consequences. Even if populations escape the immediate effects of thermal stress, latent effects can affect longer-term resilience of populations upon the return of favorable conditions.

Methods Summary

Populations. We collected four populations of *W. smithii* from each of three latitudinal regions in North America: southern (30-31°N), mid-latitude (38-40°N) and northern (46-50°N). Overwintering larvae and pupae were collected in the late winter to early spring, 2010, when the entire population was confined to pitcher-plant leaves.

Experimental conditions. All experiments were run in the leaves of intact pitcher plants provided with adult, freeze-dried *Drosophila melanogaster*, mimicking prey-capture behavior of pitcher plants in nature^{7,30}. Computer-driven multi-function environmental

rooms were programmed to provide a southern thermal year with sine-wave fluctuations in daily and annual temperature at a constant relative humidity of 80%. Day lengths were programmed to factor out any genetic differences among populations in response to day length and provided the natural progression of long days and short days that stimulate development or diapause in all populations. We were therefore able to isolate and evaluate latitudinal variation in response to the thermal year itself.

Experimental procedures. In the experimental spring, 240 first instars were introduced into pitcher plant leaves to mimic vernal oviposition in nature³⁰. Pupae were transferred to an adult cage provided with a fresh leaf for oviposition. Throughout the experimental year, pupae were counted, pupal exuviae and dead adults were sexed and counted, and eggs and subsequent hatch were collected and counted, until short days initiated diapause in the fall and long days stimulated resumed development in the spring. Fitness integrated through all four seasons (Ry) was calculated as number of first instars hatching from each population divided by 240, the initial cohort size.

Methods

Populations. We collected *W. smithii* from each of four populations of *W. smithii* within each of three latitudinal regions in North America: at southern latitudes from Florida to Alabama along the Gulf Coast (30-31°N), at mid-latitudes from Maryland and New Jersey (38-40°N) and at northern latitudes from Newfoundland, Maine, Wisconsin and Ontario (46-50°N). Seven hundred to 3,000 larvae and pupae of each population were collected in the late winter to early spring, 2010, when 100% of the population was represented exclusively as overwintered, pre-adults in the pitcher-plant leaves. All

populations were reared through two generations in a common environment (Rearing conditions) before the start of experiments.

Rearing conditions. Field-collected populations were reared to adulthood on long days (L:D = 18:6) at 80% RH in a room programmed for a sine-wave thermoperiod from 15-35°C that lagged the light cycle by 3h as in nature²⁹. The resulting larvae were synchronized in larval diapause on short days (L:D = 10:14) at 21°C and reared as above. Each succeeding stock population was reared on this development-promoting long-day and diapause-inducing short-day program. Each stock population was maintained at a minimum of 2,000 larvae. To initiate experiments, diapausing larvae from stock populations were pooled in a large pan, 2,000 were counted out and returned to stock, the remaining larvae were reared to adulthood, and their offspring used to initiate experiments. Neither experimental animals nor their offspring were ever returned to stock.

Experimental animals. All experiments were run in the leaves of intact pitcher plants provided with adult, freeze-dried *Drosophila melanogaster* mimicking the log-normal prey-capture behavior of pitcher plants measured in the field^{7,30}: Thirty first instars were placed in 20ml distilled water in a leaf of an intact pitcher plant with 38 freeze-dried adult *Drosophila melanogaster*. During each subsequent week, each leaf was fed 150, 75, and 37 flies. The initial cohort for each population consisted of eight leaves of intact pitcher plants with cohorts initiated at a rate of two per week to mimic vernal oviposition in nature (Bradshaw 1983; Bradshaw and Holzapfel 1986). Leaves on each plant were numbered sequentially. Plants were separated by population into two-plant terraria. Terraria were placed 8cm under two, 40W cool-white fluorescent lamps for the duration

of the experiment and were moved haphazardly among shelves in the room three times per week when leaves were fed and checked for pupae. Leaves were examined three days per week for pupae using a fiberoptic light. Pupae were transferred to a 4L Perspex cages equipped with a 50ml dish of distilled water for pupae, a 40 ml dish of distilled water with a freshly cut leaf for oviposition, several pesticide-free raisins for adult nutrition, and two layers of 4mm Whatman[®] chromatography paper on the bottom. Cages were examined three times per week for adult eclosion, oviposition and dead adults. At the same time, the paper on the bottom of the cage was changed and moistened with distilled water. Pupal exuvia and dead adults were sexed and counted. Eggs were transferred with 40 ml distilled water to 150 x 25 mm Petri dishes and examined after five and ten days for first instars, which were counted and used to initiate new cohorts up to 60 larvae per week. If fewer than 60 larvae hatched in a given week, smaller cohorts were established and fed proportionally less, but over the same time span, as 30-larvae cohorts. This process was continued during the summer and fall either until all larvae, pupae and adults in a population were dead or until the entire population had entered larval diapause in the fall. Larval populations were censused twice, once during the winter and two months later during the early spring. After the return of development-stimulating long days, examination of leaves and cages, sex and counts of pupae, exuviae, and dead adults, and counts of eggs and hatch resumed as above until all of the overwintering generation was dead and their offspring had hatched.

Experimental conditions: Computer-driven multi-function environmental rooms were programmed to provide a southern thermal year with sine-wave fluctuations in daily and annual temperature at a constant relative humidity of 80%. The thermal cycle lagged the

light cycle by 3h as in nature²⁹. Daily maxima and minima varied independently with a daily high of 39°C or 38°C and low of 16°C on the hottest day of the year and with a daily high of 21.5° and low of 4.4°C on the coldest day of the year, mimicking the higher amplitude thermoperiod in the summer than in the winter. Initially, day lengths were set at L:D = 18:6 to promote continuous development in all populations, regardless of their genetically determined photoperiodic response⁶. Day lengths were switched to L:D = 10:14 and then back to L:D = 18:6 to initiate and terminate diapause in all populations at the time of year they would enter and terminate diapause in nature at 30°N³⁰. We were therefore able to isolate and evaluate latitudinal variation in response to the thermal year itself.

Bridge: From Thermal Gradients to Bloodfeeding Gradients

The study of geographic variation among populations can be pursued in many other characters that evolved with different climatic gradients. The legacy of the post-glacial range expansion in the Holocene epoch has established a mosquito species that varies in a number of other characters that are directly affected by the different evolutionary ancestry of its populations. Within the species of *Wyeomyia smithii*, we are presented with a unique opportunity to study the evolutionary loss of bloodfeeding in natural populations. There is a well-documented gradient of the propensity of bloodfeeding in *W. smithii* where southern populations will take a blood meal at a low propensity, and northern populations (above 36°C) are obligate non-bloodfeeders. Because the southern populations represent an ancestral form of *W. smithii*, the evolutionary loss of biting occurred as *W. smithii* expanded its range north. There is no other mosquito species that exhibits such a character dichotomy within such a unique

system, and so I therefore set out to first determine if morphological differences in the biting apparatus caused this cline.

CHAPTER III

**VARIATION IN BITING PROPENSITY AND MOUTHPART
MORPHOLOGY OF *WYEOMYIA SMITHII* (DIPTERA:
CULICIDAE) ACROSS A GEOGRAPHIC GRADIENT**

This study was conceived of by William Bradshaw, Christina Holzapfel, Rudyard Borowczak, and Alida Gerritsen. R.B. assayed the biting propensity of mosquito populations; A.G. collected the microscopy samples and performed the imaging and analysis; A.G. and R.B. wrote the paper.

Introduction

A general trend seen in biting mosquitoes is an increase in the incidence of autogeny (the ability to mature the first egg clutch without a blood meal) relative to anautogeny (requirement of a blood meal for an egg clutch) as species move from southern to more northern latitudes (Smith and Brust 1971, Bradshaw 1980, O'Meara 1985, Clements 1992). This latitudinal increase in autogeny in a number of different species has been ascribed to various factors, such as the effects of larval and adult nutrition, insemination, and the availability of vertebrate hosts (Clements 1992, Kassim et al. 2012). A clear north-south difference in blood feeding is seen within the single species of the pitcher-plant mosquito *Wyeomyia smithii*; northern populations (above 36°N) are obligately autogenous (Smith and Brust 1971) but southern populations among the Gulf Coast and lowland North Carolina remain facultatively autogenous (Bradshaw 1980, O'Meara 1985). Southern populations of *W. smithii* can complete the first ovarian cycle

without blood feeding but require a blood meal for subsequent ovarian cycles, while northern populations are obligately non-biting for all ovarian cycles but can still produce multiple clutches of eggs (Bradshaw 1980, Lounibos et al. 1982). *Wyeomyia smithii* is unique in that it is the only species of mosquito with fully interfertile populations that is facultatively autogenous in one part of its range and is obligately non-biting in other parts (Smith and Brust 1971, Bradshaw 1980, O'Meara 1985).

Female mosquitoes have piercing mouthparts through which blood is drawn from their hosts (Gordon and Lumsden 1939, Clements 1992). The mouthparts are comprised of a complex set of structures for insertion (maxillae), salivary injection (hypopharynx) and uptake of blood (labrum) (Clements 1992). The maxillae are paired structures on either side of the labrum that are inserted into a host (Gordon and Lumsden 1939, Clements 1992). Projections known as teeth along the distal side of the maxillae serve to anchor it to the host during the blood meal (Deep et al. 2003). Hudson (1970) observed that females from northern, obligately non-biting populations of *W. smithii* appear to have retained biting mouthparts when compared with other biting species. Hudson (1970) did not compare northern non-biting populations with facultatively biting southern populations.

This study was undertaken to compare variation in biting propensity among different populations of *Wyeomyia smithii*, with the morphology of their respective mouthparts to determine if the loss of blood feeding in northern populations can be attributed to functional changes in mouthparts.

Materials and Methods

Biting propensity. Ten populations of mosquitoes were collected in 2010 from a wide geographical range that represented the full range of biting in natural populations (Fig. 1 and Table B1 [Appendix B]). The biting propensity of each population was assayed after at least two generations of lab-rearing to mitigate field effects. Populations were reared on a 18:6 light:dark cycle with a sine wave thermoperiod (high:low temperature = 15:35°C) that lagged the light cycle by 3 hours. Populations were raised in cohorts of 1,260 individuals and one cohort from each population was assayed for biting propensity. Cohorts were offered an anesthetized rat for 15 minutes three times per week starting one week after the first adult eclosion. The rat was offered at the same time of day, 6-8 hours ZT time (hours after dawn), as long as adults remained alive. Any female that landed on the rat and inserted her proboscis as evidenced by a bent labium was counted as a “biter” and removed from the cage. In no case was an uninterrupted insertion observed not to result in imbibition of blood. The number of adult females in a cohort was determined by sexing pupal exuviae. The incidence of biting was then calculated as the number of biting females divided by the total number of eclosing females.

Electron microscopy. Biting females were removed from cages and immediately fixed for imaging in a 4% glutaraldehyde solution. Females from historically non-biting populations and males were sexed as pupae and fixed once they eclosed. Non-biting females from southern biting populations were collected by offering a rat for 10 consecutive days and removing all biting females from the cage. After 10 days of offering a blood meal all remaining females were classed as “non-biting” and removed for imaging. All samples were stored in glutaraldehyde until 48 hours before imaging, at

which point they were dissected to remove the inner stylets from the labium and transferred to a 30% ethanol solution. Samples were allowed to air dry for 10 minutes before stage mounting. Images were taken on the FEI Quanta 200 Microscope in the University of Oregon’s MicroAnalytical facility. Micrographs were taken in low vacuum at 10 kV and 70 Pa.

Number of maxillary teeth was determined using the following criteria: only one maxilla per individual was counted, and all projections on the appropriate side were counted as “teeth” (Fig. 2a). Number of teeth per individual was recorded for each cohort and entered into R (R Core Team 2012) for statistical analysis.

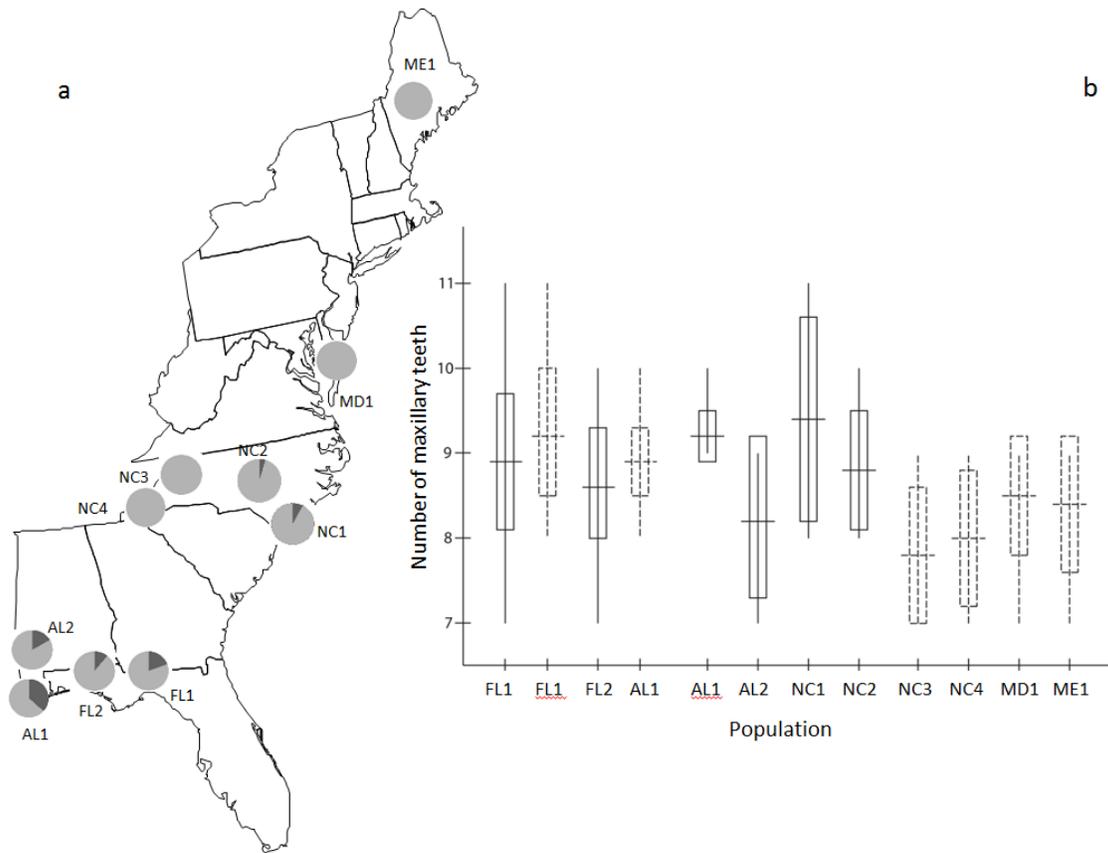


Figure 1: (A) Shows the geographical distribution and biting propensity (% of biting females) of populations sampled in this study; (B) Plot showing summary statistics for each population: horizontal lines represent mean number of teeth, closed boxes represent $\pm 2SE$, and whiskers represent the total range in the population. Dashed lines represent the range of teeth from non-biting females from either historically biting populations or northern non-biting populations.

Results

Geographical Variation in Blood Feeding. The percentage of biting females in bloodfeeding populations ranged from 4.6% to 36.7% (Fig. 1a and Table B1[Appendix B]). The highest percentages were found in the southernmost populations, with blood-feeding propensity decreasing with increasing latitude or altitude. No females from above 36°N latitude or above 200m elevation took a blood meal.

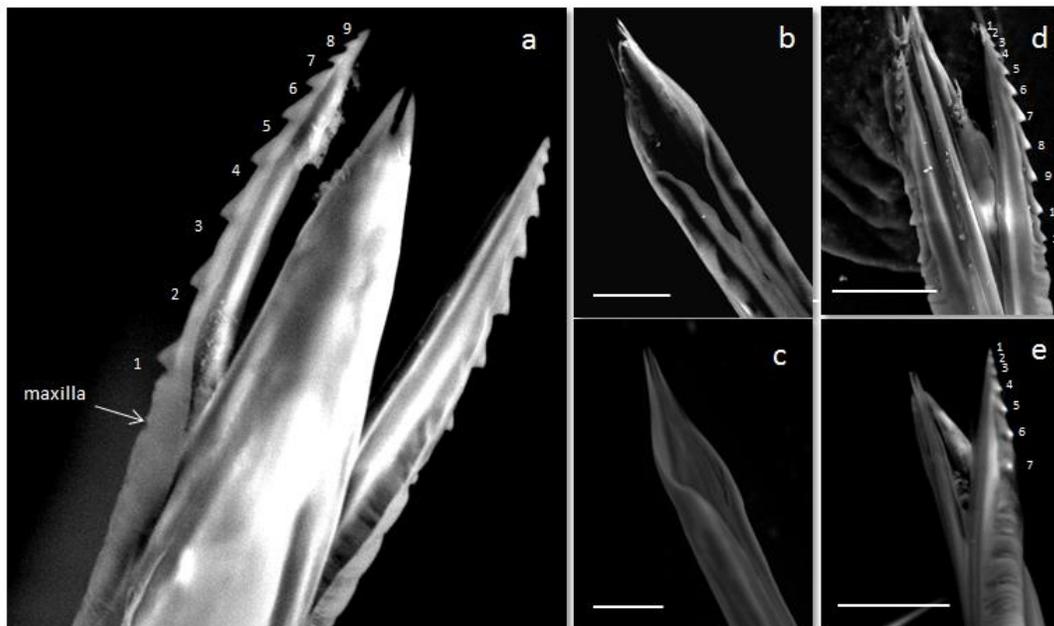


Figure 2: ESEM pictures of *Wyeomyia smithii* mouthparts, scale bars are 20 μm : (A) picture of female *W. smithii* maxillae and labrum. Each projection that counts as a “tooth” is labeled; this particular individual has 9 teeth; (B) southern male mouthparts showing lack of maxillae; (C) northern male mouthparts showing lack of maxillae; (D) southern biting female of population FL1 with 11 maxillary teeth; (E) southern biting female of populations FL1 with 7 maxillary teeth.

Geographic Variation in Mouthpart Morphology. Environmental scanning electron microscope micrographs were taken of 5-10 individuals from each population and the numbers of teeth were counted. Males from both biting and non-biting populations showed similarly reduced numbers of mouthparts (Fig. 2b-c). Among females, the number of maxillary teeth ranged from 7-11. The range of number of maxillary teeth varied among populations but the range in numbers of teeth within one southern population (FL1) was as great as within all other populations combined (Fig. 1b and Table B1 [Appendix B]). Overall, the maxillae from biting populations had more teeth than maxillae from non-biting populations, both in terms of mean number of teeth (ANOVA: $F_{1,61} = 8.24$; $P = 0.116$) and in terms of median number of teeth (Kruskal-Wallis rank sum: $\chi^2 = 6.02$, $df = 1$, $P = 0.014$). However, within the two southern populations with the greatest propensity to bite (AL1, FL1) there was no difference in number of maxillary teeth between actually biting females and females that had refused to take a blood meal (ANOVA: $F_{1,33} = 0.76$, $P = 0.39$; Kruskal-Wallis $\chi^2 = 1.27$, $df = 3$, $P = 0.736$.)

Discussion

As *Wyeomyia smithii* expanded its range to more northern latitudes after the retreat of the Laurentide Ice sheet some 20,000 years ago, it shifted from facultative autogeny to obligate non-biting, a feature that is unique within the single species of mosquito *Wyeomyia smithii*. Blood feeding has long been thought to be advantageous in that it should provide much needed proteins and lipids for the development of one or more egg clutches (Clements 1992). The impacts of larval nutrition, absence of suitable

hosts, and increased host defenses in temperate and polar regions have all been proposed as reasons for the development of autogeny (O'Meara and Evans 1973, Kassim et al. 2012). The current study was undertaken to determine if the complete loss of blood feeding for all ovarian cycles in *W. smithii* in northern latitudes could be attributed to morphological changes in the size and shape of the maxillae, which are principal mouthparts involved in blood feeding (Gordon and Lumsden 1939, Clements 1992, Wahid et al. 2003). The non-biting males from biting species regularly show reduced size and number of mouthparts (Clements 1992, Wahid et al. 2003, Wahid et al. 2007), as do males of *W. smithii* (Fig. 2b-c). Females from the obligately non-biting *Toxorhynchites* and *Malaya* have missing or reduced mouthparts when compared to biting genera (Lee and Craig 1983, Clements 1992, Wahid et al. 2007). The loss of maxillae or maxillary structures in non-biting *W. smithii* females could therefore implicate morphological changes in the piercing mouthparts as a causal factor in the evolutionary loss of the biting phenotype in *W. smithii*.

Our results showed that *W. smithii* exhibits no biting phenotype above 36°N or above 200m elevation (Fig. 1a) and that the number of maxillary teeth was significantly reduced in non-biting northern and mountain populations. However, the biting females from the southern FL1 population showed as wide a range in the number of maxillary teeth (7-11) as all other populations together (Fig. 1b). In addition, there was no significant difference in the number of maxillary teeth between biting and non-biting females within the two populations with the greatest propensity to bite. Furthermore, the non-significant trend was towards fewer teeth in the biters than in the non-biters (Fig. 1b). These results mean that the number of maxillary teeth between biting and non-biting

populations is not, in itself, an impediment to blood feeding. We therefore conclude that the reduction in number of maxillary teeth in non-biting as compared with biting populations is more likely a consequence rather than the cause of the evolutionary loss of blood-feeding in the northern and related mountain populations of *Wyeomyia smithii*.

Bridge: From Morphological Comparisons to Molecular Comparisons

In the previous chapter, we have established that morphological differences in the biting apparatus are not directly causative of the difference in bloodfeeding propensity observed within *W. smithii*. We have also established that we have reason to believe the bloodfeeding trait is under genetic control in that it is heritable and manifests at a consistent propensity even after generations of lab-rearing without bloodfeeding. Therefore, our next logical step to examine the reasons for the evolutionary loss of bloodfeeding is to approach the question from the molecular level, using the most recent advances in DNA and RNA-sequencing technology to analyze the differences between populations of *W. smithii* that have a low propensity to bite versus populations that are obligately non-biting. We are interested at what is going on at the level of gene transcription and if we can identify differential expression that could indicate what genes may be different between biting and non-biting populations.

CHAPTER IV

THE EVOLUTIONARY LOSS OF BLOODFEEDING IN NATURAL POPULATIONS OF *WYEOMYIA SMITHII*: AN RNA-SEQ STUDY

Alida T.Gerritsen, William A. Cresko, William E. Bradshaw, and Christina M. Holzapfel conceived the study and designed the experiments. A.T.G. ran the experiments and analyzed the data; A.T.G., W.A.C., W.E.B., and C.M.H. wrote the paper.

Background

Across the world, mosquitoes are vectors of a multitude of human and animal diseases, some of them extraordinarily costly in terms of human health. Mosquito-borne diseases such as Dengue and Yellow fever, Eastern and Western equine encephalitis, and West Nile Virus all have the potential to spread and cause serious health and financial consequences worldwide. Much research has been focused on vector control by eliminating mosquitoes using insecticides or eliminating their breeding grounds, but these methods are expensive and often do not eliminate them entirely. According to the W.H.O. World Malaria Report 2013 [1], malaria remains one of the most virulent of these mosquito-borne illnesses, resulting in more than 500,000 deaths per year. The *Plasmodium* parasite that causes malaria is carried by the *Anopheles* genus of mosquito and is largely successful due to its vector's affinity for human hosts [2].

Female mosquitoes bloodfeed in order to obtain nutrients to make yolk for their eggs [3]. Several species have the ability to lay multiple clutches of eggs as long as they have access to blood meals, and this repeated blood-feeding can result in the spread of

disease [3]. Mosquitoes inject saliva as an anti-coagulant into the host's body and, if they have previously fed on a host that carries a mosquito-borne illness, the next host has a chance of becoming infected [4, 5]. If a mosquito does not have a chance to repeatedly feed on human or animal hosts, the virulence of these diseases would be greatly reduced. Given that the bite of the mosquito is what spreads the disease, we propose that approaching disease control at the level of the bite has considerable promise as an alternative to insecticides or eliminating breeding grounds. Additionally, approaching the problem from the level of the disease vector allows multiple diseases to be addressed at the same time. Techniques that prevent the vector from spreading the disease would be much more effective than expending resources towards eliminating multiple species of *Plasmodium* or dealing with a rapidly mutating virus in another disease system.

Within the system of *Wyeomyia smithii*, there exists a unique opportunity to examine the cessation of biting in natural populations. *Wyeomyia smithii* is a small Culicine mosquito that spends its pre-adult development within the water-filled leaves of its host plant, the carnivorous pitcher plant *Sarracenia purpurea*. *Wyeomyia smithii* closely follows the range of *S. purpurea* along the coast of eastern North America, beginning in the Gulf Coast and extending as far northeast as Newfoundland and as far northwest as Alberta, Canada. Distinct populations of *W. smithii* have been collected and established as lab-raised populations within the Bradshaw/Holzappel lab and exhibit several different phenotypes based on the latitude from which they originate. One such differential phenotype is the complete lack of bloodfeeding in any populations found above certain latitudes: northern populations (above 36°N) are obligate non-biters [3, 6] but southern populations among the Gulf Coast and lowland North Carolina retain the

ability to bloodfeed [7-9]. Southern populations of can complete the first ovarian cycle without bloodfeeding but require a blood meal for subsequent ovarian cycles, while northern populations are obligate non-biting for all ovarian cycles but can still produce multiple clutches of eggs [3, 7, 10].

Wyeomyia smithii is unique in that it is the only species of mosquito with fully interfertile populations that can bloodfeed in parts of its range and are obligate non-biters in other parts [6-8]. This system provides us a valuable opportunity to investigate the genetic differences that have led to the evolutionary loss of bloodfeeding in northern populations, all within a single species. Because the propensity of bloodfeeding in natural populations of *W. smithii* is 38% or less, our lab has established a selection protocol to found a population of voracious biters [11]. Selection was performed by allowing females to feed on a blood source and collecting their progeny to found the next generation while maintaining a large population size to prevent inbreeding depression. The selection process is ongoing and has reached the 15th generation of progeny with a blood-feeding propensity between 55% and 60%, while the ancestral population has a propensity of less than 20% biting [11].

Physical differences in biting mouthparts have been hypothesized to be the cause of the loss of biting in northern populations [12]; however, we have previously shown that differences in mouthparts between biting and non-biting populations are not a causal factor in the evolutionary loss of biting, and physical differences do not appear to affect the propensity to bite [9]. We propose, therefore, that the evolutionary differences that resulted in the lack of biting in northern populations involve other genetic mechanisms, which could include differential gene expression and/or the loss or gain of particular

genes or genomic regions. The genetics of biting has been studied extensively in mosquitoes, and there have been a number of transcriptomic studies that compare the characteristics of biting mosquitoes that have not taken an initial blood meal with those that have [13-16]. However, those studies are necessarily limited by using species of mosquitoes in which all individuals are capable of taking a blood meal.

For this study, we sampled the mRNA expression between three populations: an obligate non-biting northern population, a southern historically biting population that is ancestral to the selected line, and the selected biting line at the time of a biting stimulus. We performed two transcriptional comparisons within this study; the first comparison was between the obligate non-biting northern population and historically biting southern population that would indicate expression differences at the population level. However, because we also wanted to identify transcripts associated with the act of biting, we also compared the transcriptional expression between the historically biting population and the selected biting line.

We hypothesized that the transcripts we identify in this study may be expressed in similar ways if the differences occurring during the evolutionary loss of biting are correlated with the transcriptional differences between biting and non-biting mosquitoes of similar ancestry. To illustrate our hypothesis, Figure 1 shows the hypothetical distribution of the expression changes that could be correlated with genetic differences between biting and non-biting populations. The red points in quadrants 2 and 4 represent transcripts that are significantly differentially regulated in the same direction across both experiments, so that genes up-regulated between the selected line and the ancestral southern line are also up-regulated in the comparison between the ancestral southern line

and the obligate non-biting northern line. Additionally, we did not necessarily expect an even distribution between the quadrants as differential expression as relates to bloodfeeding could involve significant up or down-regulation of genes, but we did hypothesize that the direction of expression would be consistent among comparisons. The blue and red arrows indicate the direction of transcriptional expression in either the southern/northern comparison (blue) or the southern/selected comparison (red).

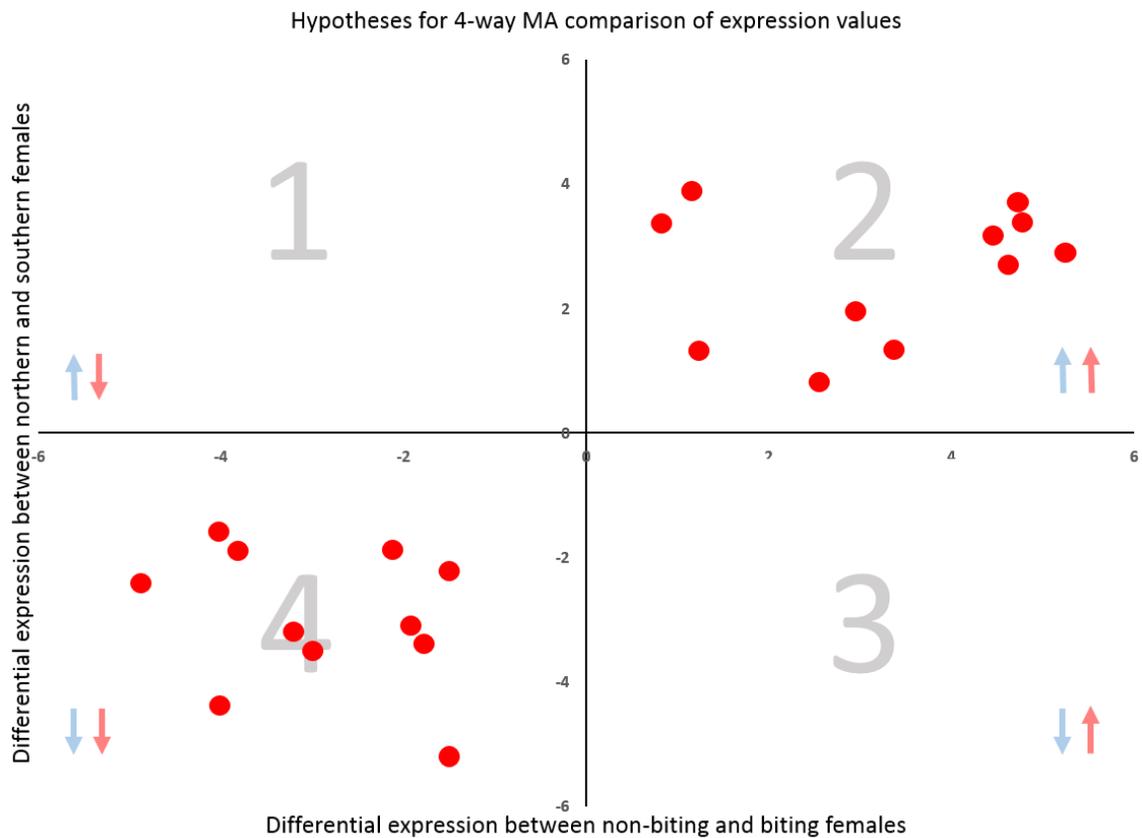


Figure 1. Hypothesized distribution of overlapping up and down-regulated genes when comparing increased biting females with non-biting females and a historically biting population with an obligate non-biting population. The distribution could be concentrated in either quadrants 2 or 4 as long as the direction of expression remains the same between comparisons.

New methodologies and increasing sensitivity of technology have made a transcriptomic analysis feasible with the small amount of RNA produced by a mosquito for a fraction of the cost of previous methods [17]. Additionally, many open-source software programs are now widely available for generating genetic resources for non-model organisms and have been used in numerous expression profiling studies [18-38]. Given the available tools, we set out to characterize the transcriptional differences that may indicate the genetic pathways involved in the evolutionary loss of the biting phenotype in the very first RNA-sequencing experiment of its kind performed on *W. smithii*. The questions we specifically address in this paper are twofold: (1) what transcriptional differences are occurring between biting mosquitoes and non-biting mosquitoes at the time of a biting stimulus? (2) What functions can we ascribe to those transcriptional differences that could be correlated with the evolutionary loss of biting?

Results

Two replicates each of three different *Wyeomyia smithii* populations were sequenced to produce 30-40 million single-end 100 base-pair reads for each library. After quality filtering and trimming the reads, the sizes of each library ranged from 28 million to 37 million reads (Table 1).

Library	Total reads	Discards	Trimmed	Total kept	% kept
WI1	33,148,877	2,118,290	3,379,742	31,030,587	93.61
WI2	34,487,148	2,383,823	3,652,571	32,103,325	93.09
WIOB1	40,818,917	2,890,071	4,170,623	37,928,846	92.92
WIOB2	33,685,455	2,309,967	3,362,773	31,375,488	93.14
KC1	33,391,905	2,359,922	3,455,628	31,031,983	92.93
KC2	30,738,892	2,015,368	3,254,750	28,723,524	93.44
Total	206,271,194	14,077,441	21,276,087	192,193,753	93

Table 1. Summary table for reads within each of the six sequenced libraries

By several estimates, 20-30 million reads are appropriate for mRNA level coverage of a transcriptome [26, 33] so an assembly was performed. Because only heads were sampled for the study, a full-coverage transcriptome assembly was not expected; however, after the stringent quality filters and optimization the assembly was of high quality according to the common metrics used to assess transcriptome assemblies [22]. The libraries were then run through a kmer-filter in *Stacks* [39, 40] to omit errors and redundancies and then pooled for the assembly. The filtered reads were assembled multiple times in *Velvet* [41] using varying kmer lengths and coverage cutoff values to obtain the optimal assembly based on number of total assembled contigs, n50, maximum contig length, and the number of reads used in the assembly (Table 2, bold). The total number of bases used in the assembly was 24,199,362; the mean contig length was 637 bp, and the number of contigs greater than 500 bp was 16,201.

Hash length	# Contigs	n50	Max length	Used reads	coverage cutoff
41	106,080	650	14,765	8,627,378	7
	85,317	678	12,455	7,711,163	8
45	84,362	690	10,412	7,591,432	7
	65,160	738	10,135	6,658,213	8
	50,435	773	10,066	5,655,955	9
	38,448	782	10,032	4,556,118	10
49	71,072	661	10,683	6,602,559	7
	52,156	722	10,683	5,573,159	8
	37,546	763	8,220	4,443,816	9
	24,691	767	7,017	3,087,899	10

Table 2. Table summarizing parameter optimization for hash lengths 41-49 and the final assembly chose for the analysis (**bold**).

Reads were aligned to the assembled reference using *bowtie2* [42] and read counts for each contig were obtained with *samtools* [43]. The raw read counts were then input into *DESeq* [44] for the differential analysis according to the negative binomial model. The first transcriptional comparison (Figure 2) was of non-biting females from the historically biting southern population (WI) and non-biting females from the obligately nonbiting northern population (KC). There were 35,534 contigs used in the comparison (Figure 2) of which 4,071 were significantly differentially expressed. Points outlined in red represent those significantly differentially regulated contigs and are corrected for false discovery rate cutoff of 0.05 by the Benjamini-Hochberg correction [44].

The second transcriptional comparison (Figure 3) was between non-biting females of the historically biting southern population (WI) and biting females that were selected from the historically biting southern population (WIOB). There were 27,814 contigs used in the comparison, of which 117 were significantly differentially expressed. Of the 117 differentially expressed contigs in Figure 3, there were 45 contigs that were also significantly differentially expressed in Figure 2.

BLAST results from the NCBI nr/nt database [45] of the 45 common differentially expressed contigs returned 28 significant identifications (E value < 1×10^{-10}) and 17 ambiguous alignments. Our null hypothesis for this experiment predicted that differentially expressed contigs would be evenly distributed through the 4-way plot, showing no clear direction or correlation of expression values. Among the 28 significant identifications, there is a significant weighting [$\chi^2(3df) = 28.71$; $P = 2.5E-06$] along the quadrant 2-4 diagonal axis (Figure 4).

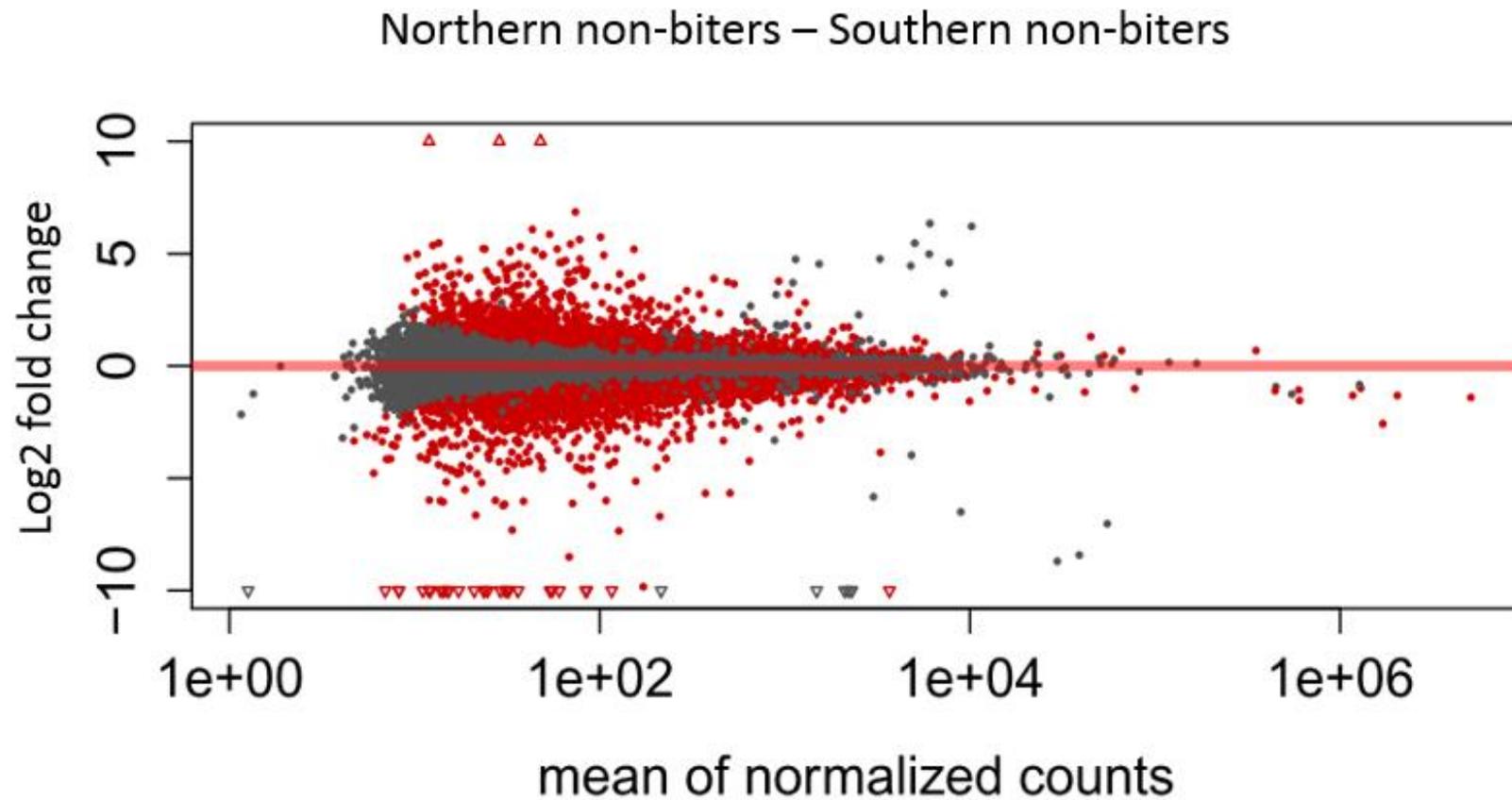


Figure 2. MA plot for obligately non-biting northern females and historically biting southern females: MA plot of logfold expression change by strength of expression showing differential expression between an obligate non-biting northern population (KC) and a historically biting southern population (WI).

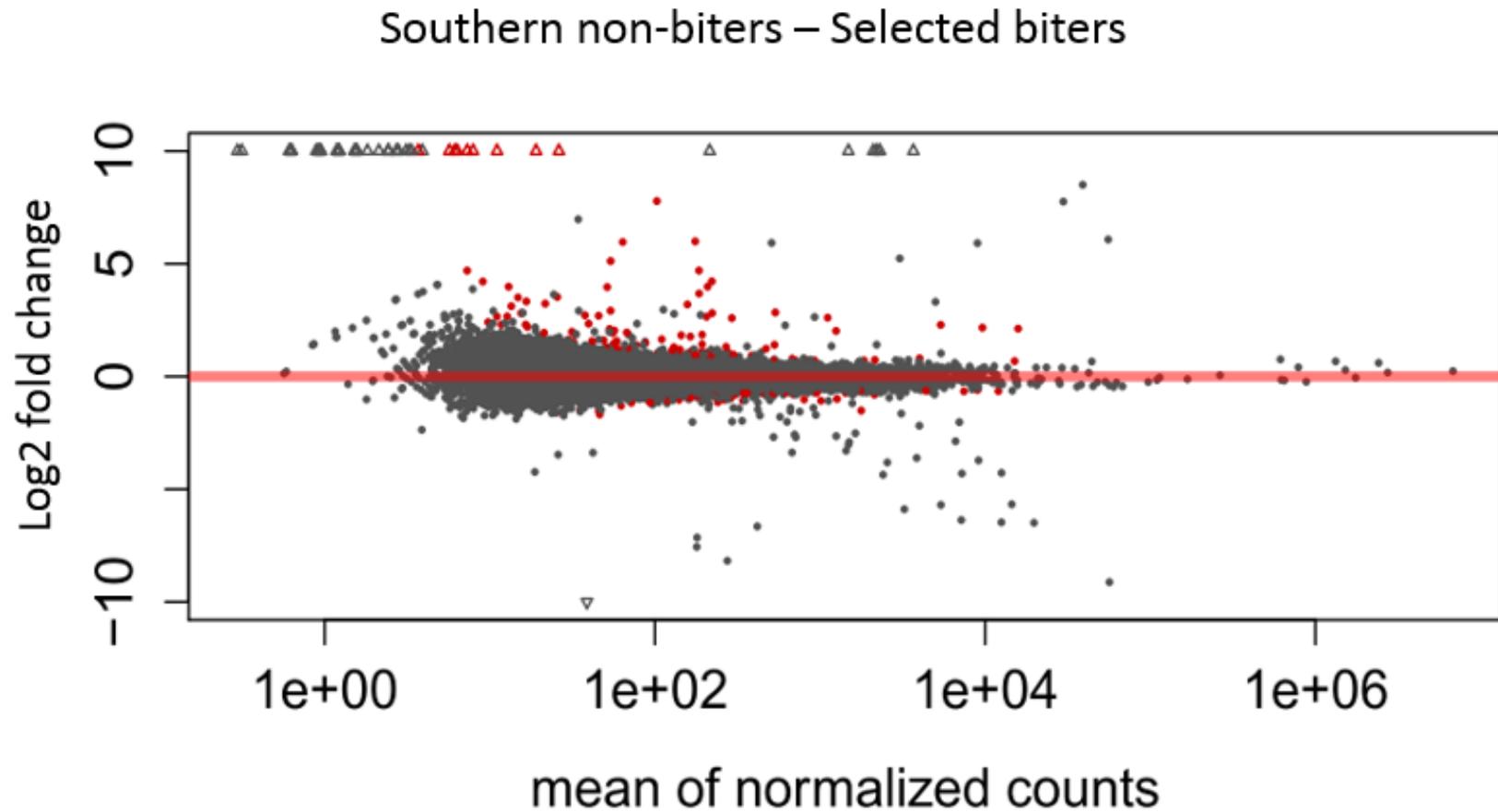


Figure 3. MA plot for historically biting females and increased biting females: MA plot of logfold expression change by strength of expression showing differential expression between a non-biting females from a historically biting southern population (WI) and biting females from a selected biting line derived from that same historically biting southern population (WIOB).

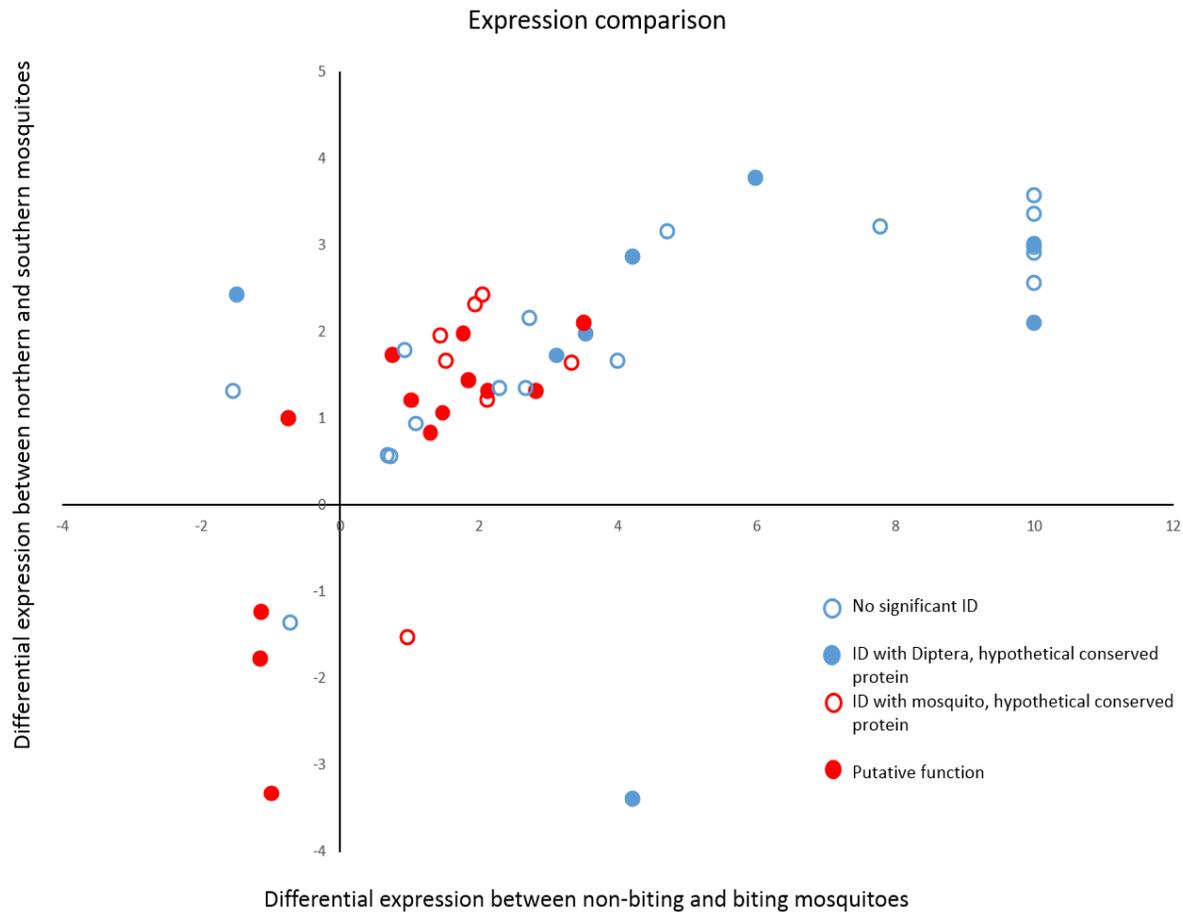


Figure 4. A 4-way expression comparison between KC/WI and WI/WIOB. Quadrants are numbered 1-4, with quadrants 2 and 4 representing the hypothesized expression patterns for the evolutionary loss of biting. Quadrant 2 shows contigs up-regulated in both comparisons, and quadrant 4 shows contigs down-regulated in both comparison. Contigs with BLAST identifications to mosquitoes are labeled.

Contigs with a positive identification that aligned to a mosquito reference are outlined by the identified gene and its function (Table 3). Only the contigs that had a clearly significant BLAST identification were investigated for function.

Quadrant	Putative Gene	Function
1	<i>Aedes</i> cytochrome P450	Oxidative enzyme
2	<i>Aedes</i> cuticle protein	Cuticle structure
2	<i>Aedes</i> neuroligin	Synapse formation and communication
2	<i>Aedes</i> “moose” LTR-retrotransposon	Transposable element, could be used to insert genes into germline
2	<i>Aedes</i> phosphatidylserine receptor*	Prevents apoptosis, could affect JNK pathway
2	<i>Aedes</i> SNMP2	Sensory neuron membrane protein in sensilla support cells
2	<i>Aedes</i> acetylcholinesterase*	Hydrolysis of acetylcholine, target of many insecticides
2	<i>Culex</i> vitellogenin A1	Yolks eggs
2	<i>Culex</i> actin	General cellular functions
2	<i>Culex</i> mitochondrial gene	From mitochondrial sequences
4	<i>Culex</i> nnp-1 nuclear protein	Important for rRNA processing
4	<i>Culex</i> fibrinogen	Cellular structure
4	<i>Aedes</i> SNMP1	Antennal specific pheromone detection

Table 3: Genes with positive BLAST identification and function.

Discussion

Mosquitoes remain the most virulent vector of numerous human and animal diseases and have even been shown to be expanding their ranges with current climate change [46]. As a result, vector control is a rapidly changing field, with new genetic control techniques being employed as an alternative to the repeated application of insecticides. The sterile male technique (SIT) [47] and the recent advances in RNA interference (RNAi) experiments in mosquitoes [48] are both examples of expanding genetic information into applicable solutions. Knowing which genes are involved in the bloodfeeding trait is of utmost importance, as it gives researchers a basis upon which they can develop alternative control methods. Within this study, we have identified several

transcripts that are differentially expressed in mosquitoes with differing evolutionary histories, and we have correlated the expression of these transcripts with the act or lack of biting. Understanding the genetics of mosquito biting is fundamental for the advancement of vector control, and our aim is to contribute information about the transcriptional differences between biting and non-biting populations.

Assembly and differential expression between populations

The differential expression analysis revealed that although there are on the order of thousands of transcriptional differences between historically biting and obligate non-biting populations separated by long evolutionary times (WI and KC, Figure 2), those differences decrease dramatically when the comparison is restricted to differentially expressed genes between biting and non-biting individuals from the same ancestral population (WIOB and WI, Figure 3). This comparison resulted in many fewer differentially expressed transcripts than previous mosquito studies comparing transcription between sugar-fed and blood-fed mosquitoes after a blood meal, especially taking into account the large number of contigs in this study relative to others [13, 14]. This relatively small number of transcripts differentiating biting mosquitoes from non-biting mosquitoes suggests there may be a small number of genetic changes that underlie differences in biting ability, but we cannot conclude anything specific until we have investigated the non-transcriptional genetic differences as well.

The 4-way expression comparison plot (Figure 4) shows a clear directionality towards a biting/non-biting diagonal with heavy weighting in quadrant 2. This fits with our hypothesis that we would be able to pick up a correlated direction of expression when comparing the evolutionary loss of biting with the act of biting, although we cannot yet

determine if any of these genes are directly related to the loss of biting. We are, however, able to assign putative functions to thirteen contigs which strongly identify with genes of known function in Diptera (Table 3). Four of those contigs in particular, detailed below, are interesting for their role in the gonotrophic cycle or in sensory differentiation.

4-way comparison transcript identifications

SNMP2 (Sensory neuron membrane protein 2) has been characterized in both Lepidopterans [49- 51] and Dipterans [50, 52, 53] as having an important role in pheromone detection. The full role of SNMPs has yet to be determined in the pathways involved in odorant detection, but the presence of this transcript indicates that sensory information may be received or interpreted differently between biting and non-biting populations.

Neuroigin, a post-synaptic cell adhesion molecule, has been studied extensively in *Drosophila* for its role in moderating social behaviors and communication signaling, and has many implications for the human autism spectrum [54, 55]. Neuroigin functions by forming dimers with another post-synaptic molecule and facilitates bi-directional trans-synaptic signaling [55]. Interestingly, it has been shown that neuroigin promotes the maturation and maintenance of synapses [55] and could have a major role in the transfer and interpretation of sensory information in mosquitoes.

SNMP1 (sensory neuron membrane protein 1) is related to SNMP2 but the two transcripts are distinct isoforms of the SNMP complex and SNMP1 is exclusively expressed in antennal neurons [50, 53, 56]. It is a required component of sex-pheromone sensitivity in *Drosophila* [53] and Lepidoptera [56], and its presence in this comparative

study suggests that could possibly be involved with host perception in addition to sex pheromone reception.

Vitellogenins are precursors to yolk proteins that provide nutrition for developing embryos in vertebrates as well as insects [57]. Mosquitoes that are able to reproduce without a blood meal will synthesize vitellogenin immediately following adult emergence [3] but mosquitoes that require a blood meal will enter a reproductive pause and will not produce vitellogenin until they receive blood [58, 59] In species that can produce multiple clutches of eggs, a blood meal is required to stimulate vitellogenesis after production of the first egg batch [58]. In *W. smithii*, we would therefore expect vitellogenin synthesis to be higher in non-biting than in biting samples because the biting phenotype was assessed during and not after the ingestion of a blood meal. That pattern is exactly what we observe, as vitellogenin 1A is up-regulated in non-biters as compared with biters (Figure 4, quadrant 2). The presence of vitellogenin indicates that biting mosquitoes are in different reproductive stages than non-biting mosquitoes and that differences within the timing of the gonotrophic cycle could be correlated to differences in ability to bite.

Biting and non-biting transcript identifications

The transcriptional comparison between non-biting females from a historically biting population and biting females from the biting selected population resulted in 117 significantly differentially expressed transcripts, 45 of which were also seen in the comparison between northern and southern mosquitoes. The remaining 72 transcripts that were not found in the north/south comparison we considered to be worth additional investigation because they are indicative of the differences involved in the act of biting.

Of these 72, 26 returned no significant identification, leaving 46 that identified within a NCBI database. Of these 46, 25 returned hypothetical conserved protein identifications, which are not useful for our purposes beyond knowing they are found in other organisms.

Overall, we found 21 annotated transcripts. There were several common regulatory transcripts such as actin, cuticle proteins, and ribosomal proteins that could not be investigated beyond their ascribed function. We also found several vitellogenin transcripts, the presence of which indicate differences in the gonotrophic cycle at the time of sampling [57-59]. Interestingly, there were several transcripts that identified with heat-shock protein 70 (hsp70) that were not found in the 4-way expression comparison and were all up-regulated within the biting/non-biting comparison. Hsp70 sequences are highly conserved; their general function is to prevent inappropriate protein folding and they are commonly expressed during stressful events [60, 61]. Their presence in this analysis indicates that there is a certain level of stress the mosquitoes were experiencing at the time of sampling, and the increased expression in the non-biting females indicates that they were reacting to that stress more than non-biting females.

Incorporating results into future work

The results from this study have indicated several transcripts that are interesting for their possible involvement in the evolutionary loss of biting. These transcripts could be a downstream effect of changes occurring between biting and non-biting populations or possibly involved in related genetic pathways. They hold tantalizing prospects for future work that could help expand the analysis beyond the limitations of this current study. Mosquito genomics in particular has been expanding recently due to the advent of NGS [17, 62-64], and the more we understand of the biological basis of biting the more

we will be able to address the problem of mosquito-borne diseases. With this study, we show that the unique and powerful system of *Wyeomyia smithii* has the potential to add significantly to the current knowledge about mosquito vectors.

It is important to note that although this study is unique, it cannot be all-encompassing due to the restricted nature of mRNA-sequencing. Because the samples were collected at the time of a host stimulus, genes that are differentially expressed between populations at other time points such as larval development would not have been captured, and we would only be able to capture the downstream effects of those differences. Additionally, profiling expression differences does not necessarily indicate the genes that are inherently different among populations, as we are only looking at the genes that are transcribed into mRNA. If a gene were not transcribed at the time of sampling, we would not be able to identify it. Any differences due to maternal effects would also be missed. Additionally, there are many genetic processes that occur pre and post-transcription that would not be captured within this study. Although we will not be able to pinpoint genetic differences that are not present in the mRNA when we sample our populations, we may be able to see downstream effects of those genes through differential expression of transcripts that are either a part of certain genetic pathways or modified in some way. Ideally, future studies would include gene mapping and genomic analyses that could incorporate pre- and post-translational genetic differences and combine them in with the transcriptomic analyses. Future studies could also include more sampling time points, especially points in the pre-adult development that could be important for determining adult biting.

The differential regulation of several sensory and reproductive transcripts in this study suggests that there are key differences between biting and non-biting populations. However, many of the expression differences we identified could be randomly correlated with our hypothesis for the evolutionary loss of biting even with our adjustment for a false discovery rate. Expansion of this analysis using more replicates for each population would increase the power of the study to parse out differences due to random population variation. An additional option for expanding this analysis would include independent biological replicates of established southern and northern populations, which is another strength of the *W. smithii* system. The addition of these populations would greatly increase the power of the comparison and give us more confidence in drawing conclusions.

Similarly to many other transcriptional experiments, we have shown correlations between gene expression and the act of bloodfeeding. To definitively be able to determine the roles of these transcripts, we would need to be able to disrupt or silence the causative genes. RNA-interference (RNAi) has been demonstrated to be an effective gene knockdown mechanism in both Anopheline [65, 66] and Culicine mosquitoes [48, 67] and would likely be the most effective method of targeting the identified transcripts. RNAi is not without its own drawbacks, but it remains a proven and effective method of knocking down alleles of interest in mosquitoes.

This study is the first of its kind to use RNA-sequencing to analyze the transcriptional expression differences between a non-biting population and a biting population of any mosquito species. Current mosquito genomics focuses on species that have to potential to spread diseases, but we have now shown that there is value in

examining a related species outside of the major vectors for a greater understanding of the evolutionary processes involved with the modification of bloodfeeding. The evolutionary loss of bloodfeeding is most likely very complex, and may involve deeper levels of hormonal and biosynthetic pathways rather than the simple loss or modification of sensory transcripts identified here. However, the transcripts we have identified could be downstream effects of other major genes that play a role in the loss of biting, and we can correlate these transcripts with the loss of the biting phenotype in northern populations. The strength of the *W. smithii* system has provided us with a unique opportunity to examine the biological and evolutionary basis for the loss of bloodfeeding in natural populations, and we have now identified many transcripts that are correlated with the loss that we can investigate in future studies.

Methods for sampling and analysis

Mosquito rearing

All collections were performed in the CT rooms in the Bradshaw/Holzapfel lab. The rooms were programmed for a sinusoidal daily temperature fluctuation with a low of 15°C and a high of 35°C and a humidity level constant between 80% and 85%. Overhead lights were on an 18:6 light:dark cycle to induce adult development in the mosquitoes. The mosquitoes were collected from three distinct populations (southern historically biting, selected biting, and northern nonbiting) as hatchlings and synchronized in larval diapause for one month until all larvae had reached the same instar, at which point the larvae were placed into dishes in groups of 35, separated into population cohorts of 1200, and exposed to long days to induce development. All populations were fed a liquid diet of

hamster food and brine shrimp once weekly and pupae were collected from the dishes every five days. Collected pupae were placed into five-gallon cages to eclose and given access to a leaf of *Sarracenia purpurea* to induce oviposition. Pupae were randomly split between two cages of each population resulting in roughly 600 individuals per cage with approximately equal sex distributions. Eggs were collected from the cages three times weekly. Adult mosquitoes were given access to organic raisins as a carbohydrate source as long as they remained alive.

RNA collection and cDNA library preparation

Laboratory rats were used as a blood source as per IACUC protocol 10-11 and 13-15. Rats were anesthetized and introduced to each cage for fifteen minutes three times per week once five days had passed since the eclosion of the first female in the cage. The cages were bloodfed only between the hours of 12PM and 2 PM to minimize the effects of circadian transcription on the analysis and the order of cages bloodfed was randomly chosen every time. Mosquitoes that landed on the rat and inserted a proboscis were labeled as “biters,” aspirated out of the cage, and flash frozen on sterile petri dishes on dry ice. Each biter collected was confirmed to have a bent labrum, the outer sheath of the proboscis that pulls back and kinks when a mosquito inserts its biting mouthparts into the host. Biting individuals were decapitated on dry ice using sterilized forceps and the heads were homogenized in 200 μ L Tri-Reagent (Molecular Research Center, catalog number TR-118). Heads were homogenized in cohorts of 25 and stored at -80°C .

To collect nonbiting individuals, a rat was introduced to the cage as above and allowed to remain in the cage for five minutes to let the mosquitoes become aware of its presence. Once five minutes had passed, mosquitoes were removed from the sides and

bottom of the cages and flash-frozen on dry ice. The mosquitoes were then sexed by dissection to confirm that only females had been collected and their heads were homogenized in Tri-Reagent and stored at -80°C.

Homogenized samples were stored at -80°C in 200 µL Tri-Reagent until all of the collections were complete. Total RNA was extracted following the Tri-reagent protocol (Molecular Research), treated with DNase to remove residual DNA (Life Technologies, catalog number 18068-015) and quantified using the Qubit fluorometer (Life technologies, catalog number Q32855). Messenger RNA was separated from total RNA using the RiboMinus Eukaryote kit for RNA-seq (Life Technologies, catalog number A10837-08) to increase the opportunity to detect rare transcripts. cDNA libraries were prepared using the ScriptSeq v2 RNA-Seq library preparation kit (EpiCentre, catalog number SSV21106), indexed using the ScriptSeq Index PCR primers (Epicentre, catalog number RSBC10948), and purified using the AMPure XP purification system (Beckman-Coulter, catalog number A63880). A total of six indexed libraries (two per population) were submitted to the core Genomics Facility at the University of Oregon to be sequenced on the Illumina Genome Analyzer II in one full lane of 100-bp single-end reads.

Read filtering and quality control

The full lane of sequencing resulted in 241,823,616 raw reads for six libraries. Illumina quality-control filtering produced 206,271,194 reads for a total size of about 20GB. The data were then run through another quality filter using the *Stacks* software [39,40] by using a sliding window technique to average the quality score of the sequence and trimming reads once they fall below 90% average probability of being correct. Out of

the 206,271,194 starting reads, 192,193,753 reads were kept and/or trimmed. The reads were then pooled for the next step of kmer filtering which also took place within the *Stacks* module. The reads were filtered using a rare filter to remove reads that are uncommonly represented that most likely were errors [20] and an abundant filter to remove redundancies. The entire dataset was normalized to coverage of 20x to make the assembly process faster and less computationally expensive. After the filters the retained dataset was 69,446,874 reads.

de novo Velvet assembly

Reads were assembled in *Velvet* [41] due to the lack of a reference genome. An optimal k-mer length was determined by varying the k-mer length from 35-51 in intervals of 2 while keeping the expected coverage and the coverage cutoff the same (20x and 5, respectively). Once an optimal k-mer length was determined, an optimal coverage cutoff was found by varying the cutoff from 5-9 while requiring the minimum contig length to be 200 bp. Assemblies were evaluated on the accuracy by assessing the following statistics: number of total contigs, n50, number of contigs above 500 bp, n50 of those contigs above 500 bp, maximum length of contigs, and the total number of reads used in the assembly [22]. The final transcriptome assembly had 50,435 contigs, an n50 of 773 base pairs, and a maximum contig length of 10,110 base pairs.

Differential expression analysis and annotation

The quality filtered reads were aligned back to the newly created reference transcriptome using *bowtie2* with allowed soft masking [42]. Each library was aligned separately, and the percentage of reads within each library that aligned to the reference ranged between 80% and 85%. The reads that did not align were excluded from the

analysis. Reads that did align were kept and the alignments were transformed into contig hit counts by using *samtools* [43]. The output files were merged into a single spreadsheet to be able to compare between replicates of sequencing libraries and input into *DESeq* [44]. The raw read counts were normalized within *DESeq* according to total library size to correct for biases for larger libraries. The historically biting southern population and the selected biting population were compared with each other, as were the historically nonbiting southern population and the obligately nonbiting northern population. The direction of the differential analysis was towards the lesser biting population, so a contig with higher expression in the southern historically biting population would be considered positive. Variances were calculated by number of counts per read within biological replicates and the data were fit to a negative binomial distribution to assess differential expression. Multiple hypothesis testing was addressed by a Benjamini-Hochberg correction and the rest of the analysis was done using the contigs that were considered significantly differentially expressed after the correction. MA plots were generated using the entire dataset while highlighting the significant differences. The quadratic plot was generated by plotting the overlapping contigs between the two comparisons by expression level.

Overlapping contigs between the two comparisons were identified using the tBLASTx database within NCBI [45] to find annotated sequences that had high sequence identity ($E \text{ value} < 1^{-10}$) within all Diptera sequences. Hits that returned E values greater than the threshold were considered “no hit” and the contig in question was classified as unidentified. The top hit within the threshold E value was kept as the most likely identification for the contig.

CHAPTER V

CONCLUSIONS

The climatic gradient established in the Holocene epoch after the most recent glacial recession has resulted in a wide range of biological variation within the mosquito species *Wyeomyia smithii*. The legacy of these geographic gradients has been shown through a clear difference in evolutionary history in both thermal tolerance and bloodfeeding propensity.

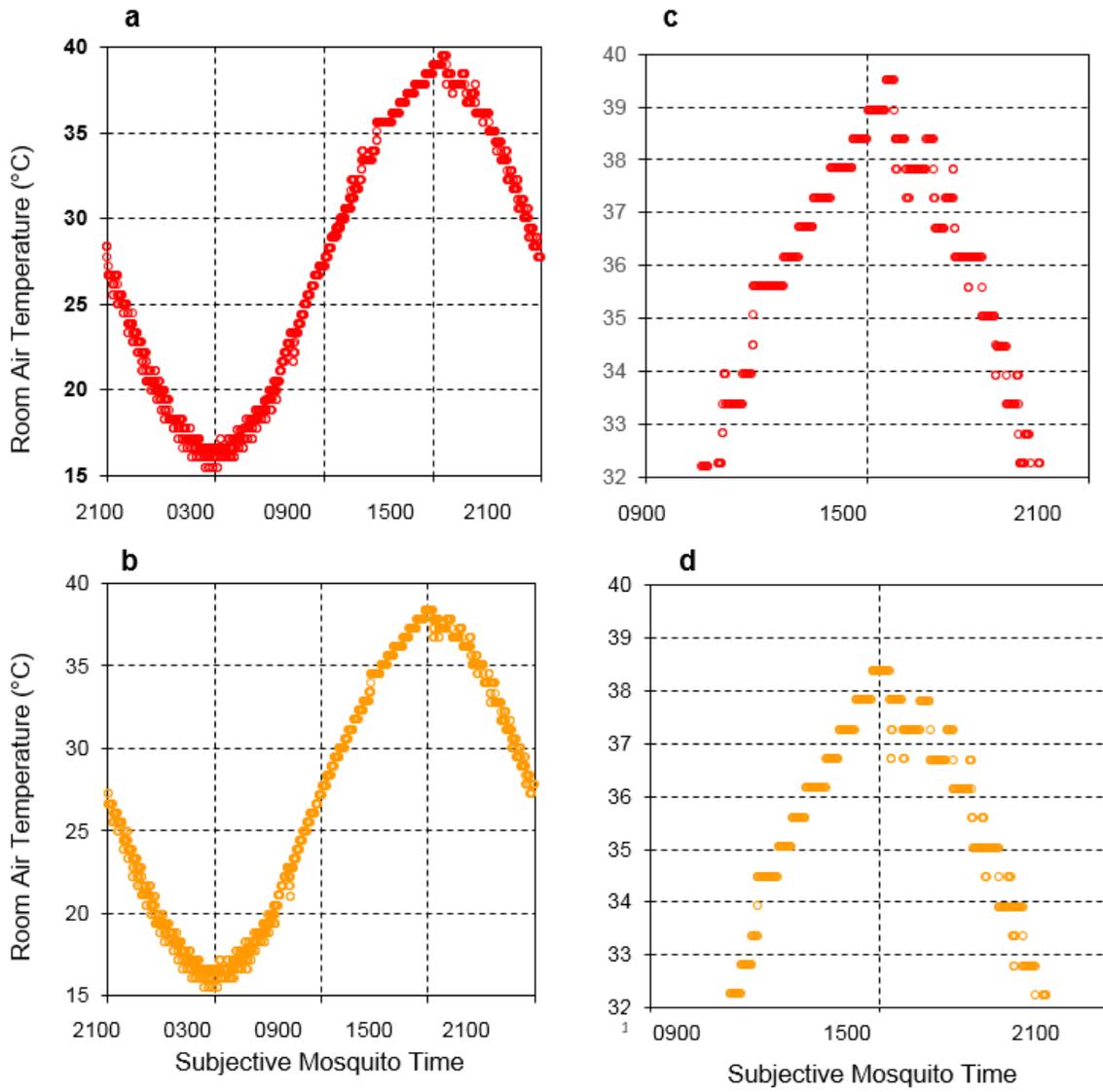
Over the past three chapters, we have examined the glacial legacy in differing thermal tolerance and propensity of bloodfeeding seen in the entire range of this organism. In the first experiment, we compared the survivorship of different populations over a narrow range of temperatures and found that there is a very narrow thermal tipping point between population viability and extinction as seen by the margin of increase of a single degree in yearly maximum temperature. The effects across latitudes are also very clear, with profound differences seen in populations with differing evolutionary history. The evolutionary loss of bloodfeeding in *W. smithii* has provided us with a unique way and a powerful system in which to examine the differences between biting and non-biting mosquitoes. Because this is the only species in which such a difference exists, we can explore the reasons for the loss in both morphological and molecular ways. We first examined the mouthparts of biting and non-biting females from different populations across the entire range using scanning electron micrographs and found that there are no morphological differences in the biting mouthparts that would form the basis for the loss of biting.

Given the unique nature of our system, we wanted to investigate this difference further and determined that a molecular approach would be reasonable. Once we had established there was not a morphological reason for the loss of biting, we examined the transcriptional differences between biting and non-biting populations at the time of a blood meal to better understand the evolutionary processes over millennial time that have left an identifiable genomic imprint. We compared the transcriptional profiles of these populations to identify differential regulation that could be correlated with the loss of biting. We identified a number of candidate transcripts that are interesting for their putative function, and we look forward to being able to study them further. By combining a number of different experimental techniques, this examination overall has revealed the fascinating impacts of latitudinal variation on the evolution of biological processes and underscores the importance of integrating evolutionary history in order to answer these important biological questions.

APPENDIX A

SUPPLEMENTARY FIGURE FOR CHAPTER II

Figure A1. Extended data



APPENDIX B

SUPPLEMENTARY FIGURES FOR CHAPTER III

Table B1. Populations of *W. smithii* sampled for the study listed by percentage of females biting by population and location.

Population	Acronym ^a	%Biting	°N Latitude	°W Longitude	m Elevation	Number ^b
ME1	KC	0	46.2	68.3°	365	5
MD1	NP	0	38.3	75.5°	18	6
NC4	DB	0	35.0	83.1°	900	4
NC3	SF	0	35.1	82.7°	670	6
NC2	GS	4.6	34.1	78.3°	20	5
NC1	SH	7.7	35.0	79.5°	107	5
FL1	WI	19.2	30.1	85.0°	10	9
FL2	BC	10.9	30.7	86.7°	10	4
AL2	CIT	16.6	31.2	88.3°	152	10
AL1	LI	36.7	30.5	87.5°	15	9
FL1	NB ^c					9
AL1	NB ^c					8

^a Acronym referring to specific populations used in earlier studies from this lab.

^b Number of females whose maxillary structures were examined and teeth counted.

^c Non-biting females from facultatively biting southern populations.

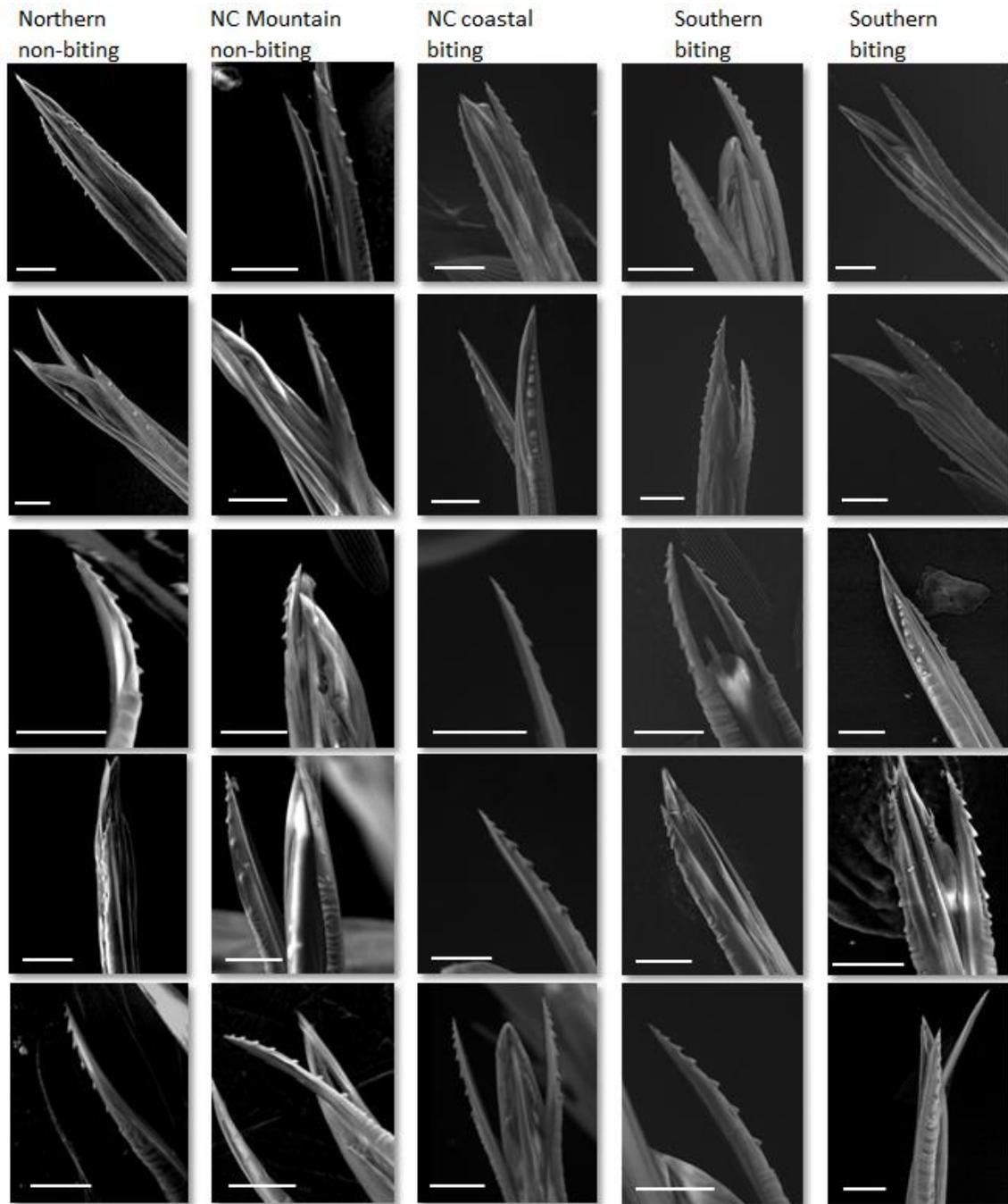


Figure B1. Sample pictures showing the variation in maxillae of females among populations.

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Chapter IV

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