

THE EVOLUTIONARY CONSEQUENCES OF THE TRANSITION TO NON-BLOOD-
FEEDING IN THE PITCHER PLANT MOSQUITO *WYEOMYIA SMITHII*

by

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DISSERTATION ABSTRACT

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Title: The Evolutionary Consequences of the Transition to Non-Blood-Feeding in the Pitch Plant

Mosquito *Wyeomyia Smithii*

The pitcher plant mosquito *Wyeomyia smithii* maintains a broad geographic range from the Gulf of Mexico to central Canada, and throughout its range is genetically and phenotypically variable, though fully interfertile. Many of the traits that vary across the broad range of this mosquito owe their diversity to selection on populations, which maximize fitness in the unique environment in which each population finds itself. While a diversity of traits vary by latitude and merit the interest of evolutionary biologists, including critical photoperiod, voltinism, and thermal tolerance, of interest in the following thesis is the variation in blood-feeding propensity within this single species of mosquito. In no other mosquito species are some populations obligate non-biters while in other populations willingly hematophagous. This thesis explores the evolutionary transition from biting to non-biting in the pitcher plant mosquito at multiple levels of biological integration, starting first by establishing a heritable basis for the transition, then moving to the fitness and life historical consequences of both the natural system and of a line artificially selected in the lab. The latter half of this thesis moves on to probe the genetic architecture underlying the shift in phenotype and ends after examining the transition to non-biting at the level of the gene using an RNA-sequencing experiment. The results stemming from

this thesis are thoroughly discussed: in short, we find that fitness differs between biting and non-biting populations, that complex genetic architectures underlie the transition to non-biting in nature, but not under artificial selection, and finally, that many candidate loci are differentially regulated in biting populations relative to non-biting populations and that these loci most often cluster with metabolic biological pathways.

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To my parents, who fostered my love for knowledge in all its forms
To my brothers, whose comradery drove me to keep exploring
To Becca, whose love is the foundation for everything I have accomplished

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CHAPTER I

INTRODUCTION: THE EVOLUTIONARY CONSEQUENCES OF THE TRANSITION TO NON-BLOOD-FEEDING IN THE PITCHER PLANT MOSQUITO *WYEOMYIA SMITHII*

The study of evolutionary biology seeks to understand and enumerate the pressures that lead to changes in organisms at multiple levels of biological integration: from the level of the gene and genome to the level of the phenotype. Our understanding of the evolutionary principles that dictate organismal change has stemmed from the study of some of the millions of species that inhabit this earth, and so it is diversity as we find it today that informs our understanding of how diversity originates. For many evolutionary questions, a system exists that is ideally suited for resolution of the question. For instance, to understand how populations diverge in sympatry, sub-species complexes that are not yet fully diverged likely lend the greatest insight into the process of diversification. Likewise, to understand the evolutionary relationships between distant relatives on the tree of life, a broader view of more diverse groups is in order.

In this thesis we are fundamentally interested in the evolution of biting in mosquitoes, of which there are over 3,500 candidate species (Harbach, 2017) that we may use to understand this medically-relevant phenotype. In all mosquito species, males do not take a blood meal. They generally subsist on nutrition sequestered in their larvalhood and from carbohydrates from plants. In most mosquito species, females take a blood meal rich in protein, which provisions their eggs; however, there is considerable variation across species in how important blood is to female reproductive success. For some species blood is required to complete ovarian development, called anautogeny, where failure to find a blood meal is tantamount to a failure to reproduce. In many other species the reproductive requirement for blood is relaxed so that females may complete ovarian development without blood, called autogeny. Though autogenous females can provision a clutch of eggs without finding a host, their fitness is generally reduced compared to individuals that successfully acquire a blood meal (Corbett, 1967; Briegel, 1993). In a

few species blood-feeding as a reproductive strategy has been lost. For example, the entire genus *Toxorhynchites* is non-blood-feeding and acquires the protein and fat necessary to generate egg batches through a penchant for carnivory as larvae. The difficulty in understanding the evolution of biting/non-biting within mosquitoes stems from species being fixed either as all females with the potential to bite, or all females non-biting. This disallows comparisons within species in which there is natural variation for biting. While interspecies comparisons between biting and non-biting taxa are possible, they are far from optimal, as differences that exist between species that have nothing to do with biting are numerous and any question that requires crossing cannot be answered. The ideally-suited species would maintain some propensity to bite in parts of its range, but have transitioned to non-biting elsewhere. Amongst the over 3,500 mosquito species there is only one that fits these criteria: the pitcher plant mosquito *Wyeomyia smithii*.

Wyeomyia smithii is a small Culicine mosquito native to the east coast of the United States and Canada; it maintains a very large latitudinal range from the Gulf coast of Florida on the southern edge to Newfoundland on the northern edge (Merz *et al.*, 2013). In all cases, the mosquito completes its pre-adult development solely within the leaves of the carnivorous pitcher plant *Sarracenia purpurea*. This advantageous, stenogamous life style provides a constant environment that reduces environmental “noise” when comparing distantly related populations. Southern populations have existed in southern locales up to the maximum extent of the Laurentide ice sheet, for hundreds of thousands of years. Northern populations are young by comparison, expanding their range through iterative founder events as the ice sheet began its retreat 20,000 years ago. Despite the time since divergence, all populations across the wide range are fully interfertile (Bradshaw and Lounibos, 1977; Armbruster *et al.*, 1997, 1998, 1999; Holzapfel and Bradshaw, 2002; Mathias *et al.*, 2006; Merz *et al.*, 2013). Genetic inference demonstrates a clear division of northern and southern clades. Phenotypically, northern and southern populations differ in several ways, but paramount in this thesis is differences in blood-feeding propensity. All southern populations maintain some females that take a blood meal prior to a second ovarian cycle. Northern populations, by contrast, have transitioned

to an obligate non-blood-feeding life style (Smith and Brust, 1971; Bradshaw, 1980; O’Meara *et al.*, 1981; Bradshaw and Holzapfel, 1983). The evolution of obligate non-biters from biting ancestors in this species provides the opportunity to probe the nature of blood-feeding and its loss in this species that is not possible in other mosquito lineages. Herein we use this natural variation in blood-feeding to examine the evolutionary ramifications of a transition away from a defining phenotype within Culicinae at multiple levels of biological integration—from fitness, to genetic architecture, and finally to the level of the gene.

Chapter II sets up the *Wyeomyia smithii* system as a model for studying phenotypic evolution. We start by quantifying the natural variation in blood-feeding propensity across a large latitudinal range in order to confirm that northern populations are obligate non-biting while southern populations retain the ancestral biting condition. We move on to demonstrate that biting in this species is heritable and under genetic control by implementing an intense selection regime for 8 years and more than 20 generations, which resulted in a voracious biting line. The line produced by selection is then used in concert with its weakly biting progenitor to understand the fitness consequences of selection on blood-feeding. Finally, we use the selected line and its progenitor in a series of crosses to understand the genetic architecture underlying the differences between high- and low-biting lines. The research in this chapter will be published as a research article. The authors will include myself, Dr. Christina Holzapfel, Dr. William Bradshaw, and Nicholas DePatie.

Within Chapter III we turn from viewing the biting phenotype through the lens of intense directional selection to the natural system in which southern biting animals transitioned to northern obligate non-biting animals through evolutionary time. We start once again by assessing the fitness consequences of a transition to non-biting by assaying replicate natural northern and southern populations. After this assessment, we look at three important life history characteristics and how they differ between these same northern and southern populations, in order to understand with greater resolution why fitness differences exist between biters and non-biters in this species. Finally, we use two northern and two southern populations to do two line crosses aimed at elucidating the

genetic architecture underlying the transition from biting to non-biting in nature. As in the previous cross reported in Chapter I, these crosses reveal the composite genetic effects (additivity, dominance, maternal and epistatic effects) leading to the difference in biting propensity between geographically distinct populations. The research in this chapter will be published as a research article. The authors will include myself, Dr. Christina Holzapfel, Dr. William Bradshaw, Mary Wood, Nicole Kingsley, Caitlin Nichols, and John Jorgensen.

Chapter IV shifts our focus to a finer level of biological integration: that of the gene, wherein we ask what genes are associated with the transition from biting to non-biting in this species. To probe at the level of the gene we use RNA-sequencing to generate millions of transcripts which are assembled into contiguous sequences and a transcriptome. Transcriptome-based approaches have become common as sequencing technologies have become more available and less expensive. The objectives of this study are three fold. First, because *Wyeomyia smithii* does not yet have a sequenced genome, we aim to create a high quality transcriptome representing all of the messenger RNA being transcribed at the moment of sampling. Second, we perform differential analyses using contigs from the transcriptome and transcript counts to assess the degree to which transcripts between different lines and treatments are being differentially regulated. Finally, to understand where differentially expressed contigs are acting in molecular pathways, we use KEGG pathway analysis, which annotates contigs with gene names and places them into biologically meaningful pathways. Our study uses a two-comparison system that refines the number of candidate causal and consequential genes. We first compare a northern non-biting population with the line selected for biting to reveal the many regulational differences that exist between northern and southern populations. Next, in order to refine our search to only those loci that are involved in biting, we compare the line selected for biting to its reluctant to bite progenitor population. The most likely genes are then assumed to be those that are differentially expressed in both comparisons and regulated in the same direction (up or down). The research in this chapter will be published as a research article, the experiment of which was conceived by myself, Dr. Christina Holzapfel, Dr. William Bradshaw, and Dr. Alida Gerritsen. The

authors will include myself, Dr. Alida Gerritsen, Dr. Christina Holzapfel, Dr. William Bradshaw, Allison Fuiten, and Ryan Meng.

The research presented in this thesis is motivated by the fact that blood-feeding in mosquitoes incurs an enormous cost to both humans and other animals (World Health Organization, 2016) around the world via their ability to vector diseases. Researchers continue to advance our understanding of medically-relevant traits and inform those within the research and medical community on how best to approach and combat vector-borne disease transmission. Within the *Wyeomyia smithii* system, evolution has offered us the unique opportunity to understand the consequences of biting at multiple levels of biological integration by turning off blood-feeding behavior in northern populations while maintaining it in southern relatives. Harnessing this system will add both to the canon of evolutionary thought and to medical innovations in hopes that someday the burden of mosquito-borne disease is no longer felt by the inhabitants of Earth.

CHAPTER II

GEOGRAPHICAL VARIATION, GENETIC ARCHITECTURE, AND

THE FITNESS CONSEQUENCES OF SELECTION IN *WYEOMYIA*

SMITHII

William E. Bradshaw, Christina M. Holzapfel, and Rudyard J. Borowczak conceived of the work and designed the experiments presented in this chapter. R.J.B, Mary Wood, Nicholas DePatie, Nicole Kingsley, Alvin Lambright, Jackie Houser, and Emily Hudson performed the experiments. R.J.B, W.E.B., C.M.H., M.W., and N.D. performed the analyses, and R.J.B., W.E.B., and C.M.H wrote the paper.

Introduction

To ask and answer almost any evolutionary minded question, especially those directed at a particular trait, at least two basic conditions must be met: variation must exist for the phenotype of interest and that variation must be heritable. Further stipulations apply when contrasting drift versus selection, but a reasonable starting point for any inquiry establishes these first two points. Previous work in the *Wyeomyia smithii* system has suggested that at least the first condition is met for this species. Smith (1902) first suggested that northern populations ($\geq 38^\circ\text{N}$) of *W. smithii* were non-biting mosquitoes. Since Smith, a number of other researchers have identified northern populations as obligately non-biting (Smith and Brust, 1971; Istock *et al.*, 1975; O'Meara *et al.*, 1981; O'Meara and Lounibos, 1981). However, no in-depth work has been done previously to articulate the variation in biting propensity among southern populations. In the following work we census a broad range of southern and northern populations for their propensity to take a blood meal to understand the diversity and degree of variation in this trait across a large geographic gradient in both major clades of *W. smithii*. While a demonstration of variation for the blood-feeding trait is useful, variation in and of itself is does not lead to evolutionary change. To be of evolutionary interest, diversity also must be demonstrated to have an underlying genetic basis. Following the census of blood-feeding propensity among populations, we implemented a selection regime aimed at increasing the blood-feeding propensity within a population native to Florida to determine if blood-feeding in

this species is under genetic control, and thus practical to evaluate in later experiments from an evolutionary perspective.

Selective regimes are useful in demonstrating the heritability of a trait, but the product of selection itself is of interest in answering questions regarding the nature of blood-feeding and its loss. In the second half of this chapter we use the selected line and its progenitor population to ask and answer two questions related to the blood-feeding phenotype: (1) what are the fitness consequences of selection on blood-feeding; and (2) what is the genetic architecture underlying the difference in blood-feeding propensity between the selected line and its progenitor. In both cases using the selected line and its progenitor are especially useful as they provide a relatively constant genetic background, reducing noise caused by genetic differences unrelated to the biting trait. However, any insight gained using the selected line must be understood in the context of artificial selection and not evolution in nature.

Methods

Standard Rearing Protocol

Herein a standard rearing protocol is described and used throughout experiments in this thesis. Populations were maintained as diapausing larvae on an 8:16 light:dark cycle at 21°C, and reared on a typical 18:6 light:dark cycle using a sinewave thermoperiod with a maximum temperature of 32°C and minimum temperature of 15°C which lagged the light cycle by 3 hours. Relative humidity was programmed for a constant 80%. Larvae were fed *ad lib.* a 4:1 mixture by volume of ground freeze-dried brine shrimp (San Francisco Bay Brand, 8239 Enterprise Drive, Newark, CA 94560, USA) and guinea pig chow (Geisler Guinea Pig Chow, Sergeant's Pet Care Products, 10077 S 134th St, Omaha, NE 68138, USA) once a week. Adults were offered a continuous supply of organic (pesticide free) raisins as a carbohydrate source. Adult cages were constructed from a 15L food-safe bucket. The cage was fitted with two mesh sides and a mesh top to easily observe mosquitoes. A freshly opened leaf from a host plant was placed in each cage to stimulate

females to lay eggs. Before experimentation larval populations were randomized by collecting all individuals of a generation into a communal container followed by redistribution of all animals into dishes of 35 individuals.

Assay of Natural Variation in Biting Propensity

Populations of mosquitoes were collected from a wide geographical range that represented the full spectrum of biting in natural populations (Figure 2.1 and Table S1). The biting propensity of each population was assayed after at least two generations of lab-rearing to mitigate field effects. To determine incidence of biting, ≥ 390 individuals were reared to adults as above. The number of eclosing females was scored by sexing pupal exuviae (pupal cuticle). Starting at first eclosion, adults were offered a rat anesthetized with a ketamine/xylazine cocktail (IACUC approved protocol 13-15RA) for 15 minutes three times per week between 1200-1400 subjective time (25-30°C). Any female that engorged blood was scored as a biter, removed from the cage, counted, and discarded. When females aggregated on the host, any female with a bent labium was scored as a biting individual and was removed from the cage. The incidence of biting was then calculated by dividing the number of biting females by the total females that eclosed.

Selection

Selection for biting began using ~14,000 individuals from a low-biting population in northern Florida (WI, Table S1). The environment and protocols used for selection were as above except biters were removed from their cage and placed into a separate “biting” cage with supplemental males from the same generation of the selected line. All hatch from the biting cage were used to generate the subsequent generations. Initially, hatch from biting females were not sufficient to maintain a line able to replace itself exclusively from biting individuals. In this situation, we used the abundant hatch generated by females from the same generation, but before they bit (pre-biters), in order to augment the selected line. This protocol was followed until the selected line could sustain itself

($R_0 > 1.0$) exclusively from biting females in the 7th generation of selection. Thereafter, hatch in excess of 5,000 were used in experiments. Through all generations of selection, including those that were not offered a host, hatch were placed on short days (L:D=10:14) at 21°C to synchronize each generation and to mitigate inadvertent direct selection on development time, generation time, or the timing of reproductive allocation. After adults of a given generation had died, their offspring were transferred to long days and reared to adulthood, as above.

Fitness Assay

Populations used for fitness analysis consisted of cohorts of 980 newly emerged larvae collected on a single day (all cohorts emerged from eggs over less than a 2 day period) and reared using the same conditions as described in the standard rearing protocol section above, but with a maximum temperature set to 35°C. Eggs, first instar hatch, pupae, and pupal exuviae were collected and counted three times a week. Because of rapid decomposition, deceased adults were collected and sexed daily for the duration of the experiment. Treatments involving a host for blood-feeding followed the protocol as in selection, except biters were not removed from the cage. To score incidence of biting, separate cages of blood-fed treatments were run in parallel with the fitness assay and incidence of biting was calculated as above. As a composite index of fitness incorporating age-specific survivorship and fecundity, we used Laughlin's (1965) capacity for increase:

$$r_c = \ln(R_0)/T,$$

where $T = \sum x H_x / \sum H_x$ and H_x = number of offspring hatch counted at age x .

Line Cross Analysis

Rearing: All populations were reared using the rearing protocol described in above experiments. The parental populations while in the larval stage were kept in diapause in a short-day room. To stimulate resumed development, the larvae were placed in a long-day room with an L:D = 18:6 hours. This amount of light is interpreted by all populations of *Wyeomyia smithii* as an unambiguous long-day, triggering the resumption of development.

The room varies the temperature following a smooth sine wave with a maximum of 35°C and a minimum of 12°C that lags behind the light cycle by 3 hours.

While exposed to long days, the larvae were fed on a weekly basis as described above. After approximately two weeks, the larvae began to pupate and were collected from the larval dishes every Monday, Wednesday, and Friday. The pupae were separated by sex, counted, and then placed into 75 ml pupal dishes with distilled water at a density of 50 per dish. Exuviae were collected every Monday, Wednesday, and Friday. The sex ratio of the mosquitoes in each cage was determined from the collected exuviae. Adult cages were constructed from a 15 L food-safe bucket. Every Monday, Wednesday, and Friday the eggs were collected into a large petri dish (source dish) and labeled appropriately with the population and date of collection. The number of eggs was counted by visual inspection before the source dish was stored in the short-day room. After 5 days, the hatched larvae were collected from the source dish and placed in groups of 35 into clean dishes with 1.08×10^{-1} mg/ml tetracycline water and dilute larval food. The larvae were stored on short days until the parental generation stopped laying eggs. During storage, the larvae were fed *ad lib* larval food weekly.

Data Generation and Analysis: The line cross involved two generations following the standard mosquito rearing protocol to produce the eight experimental F2 populations of varying genetic composition derived from the selected line and its weakly biting progenitor population. The cross is summarized in Figure S1. The final generation was raised following the standard mosquito rearing protocol with two modifications: a biting assay was conducted and eggs were not collected. An anaesthetized host (*Rattus norvegicus*) was offered for fifteen minutes between 1200 and 1400h every Monday, Wednesday, and Friday. The biting females, defined as having blood visible in the abdomen, were counted and discarded. Propensity to bite was scored as:

$$\text{Propensity} = \text{number of biting females} / \text{number of eclosing females}$$

We used a Joint-Scaling test (see Mather and Jinks, 1982) to sequentially determine the role of additive, dominance, maternal, and epistatic effects based on the biting propensities. The Joint-Scaling test evaluates the goodness of fit between the data and progressively

more inclusive models of genetic architecture using generation means and error variances as input. After confirming sufficient sample size to use the normal approximation for a binomial sample, the error variance of the frequency, p , was calculated as:

$$\text{Error variance } (p) = p(1-p)/n$$

In the equation, p is the proportion of eclosed females that bit and n is the total number of eclosed females. The Joint-Scaling test uses the χ^2 statistic to test goodness of fit for a given model of genetic effects. A significant χ^2 indicates that the model is not sufficient to explain differences among the descendent generations and a non-significant χ^2 value indicates that the model is sufficient. For example, if all points fell on the lines representing the additive expectations in Figure 2.2, a chi square test of a simple additive model would result in a non-significant p-value and thus the acceptance of the model. Model testing is sequential in that we test a simple additive model first, and if that model is sufficient we stop. If it is not, we test for a slightly more complex model of additivity and dominance. This process continues incorporating maternal and various forms of epistasis until a model cannot be rejected, at which point we accept the model.

Results

Geographical Variation

The incidence of biting declined from an average of 21% along the Gulf Coast, 5% along the Carolina Coast, to 0% at higher latitudes and altitudes (Table S1). As in the cages in which biting was observed, hosts were observed continuously in the 0% biting populations, where no female was seen with a kinked labium or with blood in her abdomen. In cages where southern females ingested blood, dark red-black splatters from excreted hemoglobin were routinely observed on the absorbent white cage floor; no such splatters were ever observed in the cages with 0% observed biting. These results support earlier reports that no females from populations at latitudes $\geq 38^\circ\text{N}$ or altitudes ≥ 600 m have ever been observed to bite (Smith, 1902; Smith and Brust, 1971; Istock *et al.*, 1975; O'Meara *et al.*, 1981; O'Meara and Lounibos, 1981).

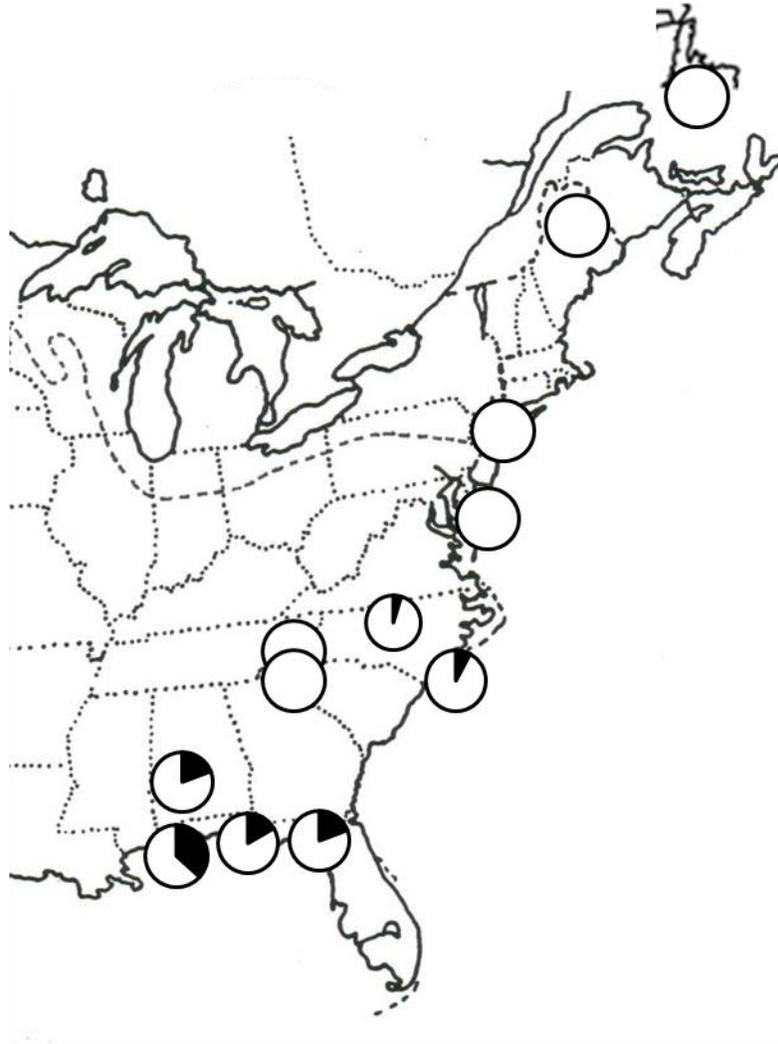


Figure 2.1 Natural variation of female biting propensity in the mosquito *Wyeomyia smithii*. Pie charts represent populations assayed for biting propensity, black shading shows the proportion of females biting during assay.

Response to Selection and Fitness Consequences

In response to selection on blood-feeding, the incidence of biting rose from 19% in the founding population (Table S1, WI) to 53% and 84% of adult females after 11 and 14 generations of selection, respectively. Initially, the incidence and fecundity of blood-fed females were not sufficient to replace the parent population. A replacement rate of

>1.0 occurred in the seventh generation of selection and a replacement rate of 2.34 from blood-feeding alone in the eighth generation of selection.

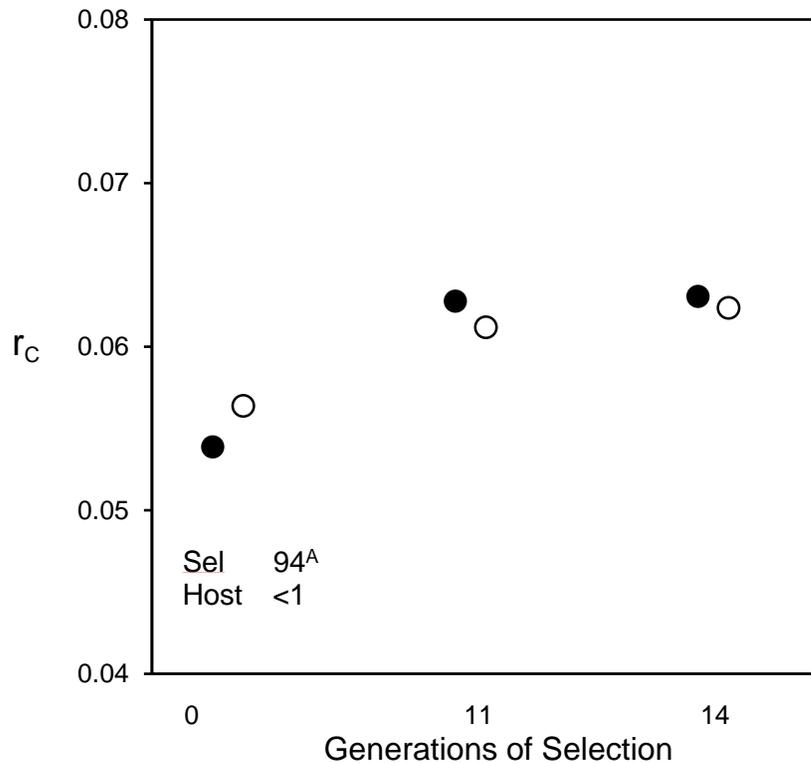


Figure 2.2 Fitness consequences of direct selection on blood-feeding. X axis shows generation of selection, Y axis shows capacity for increase. Closed and open dots represent treatments offered and denied a host respectively. Inset shows the variation explained by selection (SEL) and host availability (Host). ^A $P=0.062$.

Line Cross Analysis

Additive genetic effects are an important source of genetic variation in determining the difference due to biting between selected and non-selected lines. However, they are unable to exclusively explain the variation generated by all crosses ($\chi^2 (5) = 12.521, P = 0.028$, Figure 2.3.). Coupling dominance and additive effects is also unable to adequately explain the variation, but by incorporating maternal effects into an additive-dominance model we are unable to reject the model ($\chi^2 (4) = 12.242, P = 0.076$, Figure 2.3.). However, retaining only additive and maternal dominance effects (dropping dominance

and maternal additive effects) yields a model that better fits the data ($\chi^2(4) = 6.078$, $P = .196$, Figure 2.3). Thus, differences in biting propensity between the selected line and its weakly biting progenitor are likely due to the action of both additivity and maternal dominance.

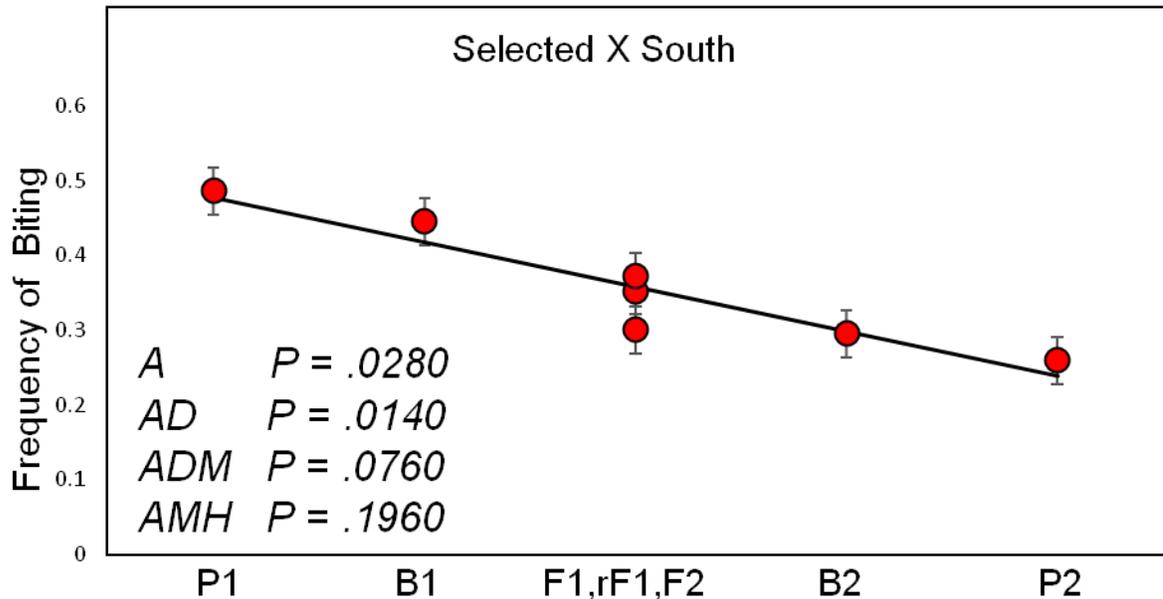


Figure 2.3 Results of the line cross using the line selected for biting and its weakly biting progenitor. X axis labels are generations of varying genetic ancestry due to the crossing design and in order are; the selected line parent, the backcross to the selected line parent, the F1 offspring between the selected line parent and the progenitor population parent, the reciprocal sexes relative to the former cross, the F2 offspring, the backcross to the progenitor population, the progenitor population parent. The Y axis gives frequency of female biters in each of the crosses (denote by red dots). Solid line represents the additive expectation. Insets show the goodness of fit to simple additive (A) additive/dominance (AD) additive/dominance/maternal (ADM), and additive/maternal dominance models (AHM). P-values below .05 demonstrate a significant departure from the model expectations, thus a rejection of that model as adequate to explain genetic differences between lines.

Discussion

For most people, biting and mosquitoes are intimately linked. It is the phenotype that defines the mosquito family Culicidae in most of our minds and is the reason that many can identify a mosquito from a lineup of insects but would be hard pressed to identify a

midge, gnat, or most other groups that do not have a proclivity to imbibe blood. Biting is extremely common amongst mosquito taxa, but there are exceptions. The genera *Toxorhynchites* and *Malaya* do not blood-feed and accumulate protein for egg production through other means (carnivorous larvalhood and ant regurgitation, respectively). Morphological and genetic analyses indicate that biting is the ancestral condition in the family Culicidae due to the placement of the anautogenous clade Anophelinae near the root of the tree (Harbach and Kitching, 1998; Reidenbach *et al*, 2009). The loss of biting in clades distributed across the Culicidae phylogeny, whose environments and morphologies are highly variable, suggests that the biting trait within mosquitoes is under genetic control, and the positive response to selection on autogeny and anautogeny in other mosquito species lends further credence to this line of reasoning (Eberle and Reisen, 1986). To establish that studies aimed at understanding biting and its loss within *Wyeomyia smithii* are of evolutionary relevance, we directly tested the hypothesis that biting is heritable. Furthermore, to articulate how the biting phenotype varies across *W. smithii*'s latitudinal range, we assayed populations from the Gulf coast of Mexico to Newfoundland, Canada, for their propensity to bite under identical environmental conditions.

Response to selection (Figure S1, Table S1) and geographic variation in propensity to bite (Table S1) demonstrate that blood-feeding is a highly heritable trait and has undergone evolution during the northward dispersal of *Wyeomyia smithii* in North America. While discrete estimates of heritability may be useful, they are difficult to make for two primary reasons: (1) biting is a female-only trait, but *W. smithii* is a strictly sexual reproducing species and (2) males contribute biting alleles to their daughters (as will become clear in line crosses done between non-biting females and males from biting populations later in this thesis). Estimates of heritability are difficult because of the nature of the system which requires information from both parents, e.g. mid-parent on average offspring regression, breeder's equation. Nevertheless, clear response to selection demonstrates this trait is under genetic control in this species. Diversity in biting propensity is widely variable across southern populations; however, through evolutionary time, northern populations have become fixed for non-biting as suggested by previous

work (Smith and Brust, 1971; Istock *et al.*, 1975; O'Meara *et al.*, 1981; O'Meara and Lounibos, 1981). Southern populations range in their propensity to bite from 4.6% of females in the Green Swamp of North Carolina to 36.7% in Southern Alabama (Table S1). Variation amongst populations raises the question of why southern population have not fixed in one direction or the other.

Among a variety of mosquitoes, blood-feeding individuals tend to achieve greater reproductive success than individuals from the same population that take a smaller number of blood meals or mature a batch of eggs without biting (Corbet, 1967; Spielman, 1971; O'Meara, 1985; Briegel and Hörler, 1993). If blood-feeding in *Wyeomyia smithii* is a mechanism underlying increasing fecundity, fecundity should be higher in the line selected for biting than in its ancestral, low biting population. However, variation within southern populations cautions any generalization between fitness and blood-feeding within this species. To tease out the effect of blood-feeding on fitness, we experimentally assessed fitness as measured by capacity for increase (Lauglin, 1965) in the selected line at various generations in which propensity to bite differed, but genetic background remained constant. The results of this experiment are shown in Figure 2.2. While it is tempting to conclude that selection on biting had no effect on fitness in this species, to do so would be at the risk of type II error, as selection explains the majority of the variation in fitness and $P=.062$. Therefore, we cannot say definitely that there is or is not an effect of selection.

In our final experiment of this chapter we examined a different level of biological integration; from the phenotype to the genetic architecture underlying differences between the line selected for biting and its progenitor. The variation in biting propensity within southern populations is interesting, as within-population estimates never rise above even 50% of females biting. This observation demonstrates that variation in this trait exists not only among southern populations but also within southern populations. A heritable trait showing variation within a population can be evaluated using various crossing regimes to estimate the composite genetic effects underlying the difference in the trait of interest. Line cross analysis has been used on a variety of organisms in past studies to articulate the genetic architecture of traits of interest including within the

mosquito *Wyeomyia smithii* (Lair *et al.*, 1997; Armbruster *et al.*, 1997). Our line cross (see Figure S2 for crossing design) utilized the line selected for blood-feeding and its weakly biting progenitor to reveal the genetic architecture of the biting phenotype under a near constant genetic background. The results of these crosses are shown in Figure 2.3 and demonstrate that selection on biting has acted on relatively simple modes of gene action, namely additive and maternal dominance effects. Neither dominance nor epistatic effects are necessary to explain the difference in biting propensity due to selection. Our results are largely in line with what evolutionary theory predicts in terms of the complexity of architectures underlying recently diverged populations (in this case 10 generations of selection within the lab) as well as with studies done using *W. smithii*. Lair *et al.* (1997) used Line Cross Analysis to show that the likelihood of observing non-additive effects underlying phenotypic differences in critical photoperiod increased as a function of geographic and evolutionary distance between the crossed populations of *W. smithii* in question. Past studies have shown that phenomena resulting from epistasis (such as outbreeding depression) are more likely to occur in distant relatives, while additive and dominance effects are generally responsible for differences observed between more closely related individuals (Carson and Templeton, 1984; Edmands, 1999; Demuth and Wade, 2007). While our results are in line with theory, it is important to remember that this suite of crosses does not represent evolution in nature. To test rigorously evolutionary predictions, natural populations that have undergone evolution in their natural environment need to be the center of questioning. In the following chapter we will take just this approach.

Bridge: From the Consequences of Selection on Blood-feeding to the Consequences of Natural Variation.

The uniqueness presented by the *Wyeomyia smithii* system is the natural transition from biting to non-biting through evolutionary time. In no other mosquito species of which we are aware exists a latitudinal gradient in which on one end of the gradient populations willingly take a blood meal and on the other end have completely transitioned away from blood-feeding. The previous chapter sought to articulate geographic variation in blood-feeding propensity and to validate the underlying genetic control of the phenotype. The following chapter concerns itself with the evolutionary consequences of the transition away from blood-feeding using natural populations. It provides insight into the forces leading to the loss of blood-feeding in this species. Herein we ask what fitness consequences are associated with the transition from the southern blood-feeding phenotype to the northern obligate non-biting phenotype. Following this assay, we look to understand the life historical differences between natural populations that underlie fitness differences, and finally we use line cross analysis to probe the genetic architecture responsible for the differences between southern biting and northern non-biting populations.

CHAPTER III

EVOLUTION OF BLOOD-FEEDING IN NATURAL

POPULATIONS: GENETIC ARCHITECTURE AND FITNESS

CONSEQUENCES OF THE TRANSITION TO NON-BITING

William E. Bradshaw, Christina M. Holzapfel, and Rudyard J. Borowczak conceived of the work and designed the experiments presented in this chapter. R.J.B, Mary Wood, Nicole Kingsley, Caitlin Nichols, and Jon Jorgensen performed the experiments. R.J.B, W.E.B., C.M.H., M.W., C.N., and Nicholas DePatie performed the analyses, and R.J.B., W.E.B., and C.M.H wrote the paper.

Introduction

Three genera of mosquitoes (*Toxorhynchites*, *Malaya*, *Topomyia*) never bite (Downes, 1958; Foster, 1995; Rattanarithikul *et al.*, 2007; Wahid *et al.*, 2007; Miyagi *et al.*, 2014; Zhou *et al.*, 2014). There also are entirely non-biting species in otherwise biting genera (O'Meara, 1985b), and many species that may or may not take a blood meal (bite) for the first ovarian cycle, but require a blood meal for the second and subsequent ovarian cycles (Spielman, 1971; Rioux *et al.*, 1975; O'Meara, 1985a). However, there is only one species of mosquito that bites in one part of its range and is obligately non-biting in the rest of its range: the pitcher-plant mosquito, *Wyeomyia smithii* (Smith and Brust, 1971; Bradshaw, 1980; O'Meara *et al.*, 1981; Bradshaw and Holzapfel, 1983). All populations of *W. smithii* are fully interfertile regardless of geographic origin or propensity to bite, and northern, obligately non-biting populations are derived from more southern, biting ancestors (Bradshaw and Lounibos, 1977; Armbruster *et al.*, 1997, 1998, 1999; Holzapfel and Bradshaw, 2002; Mathias *et al.*, 2006; Merz *et al.*, 2013). In this chapter, we are first concerned with the fitness and life-historical consequences of the evolutionary transformation from a blood-feeding to a non-biting lifestyle in *W. smithii*. We then shift our discussion down a level of biological integration to the genetic architecture

underlying the difference in blood-feeding propensity between southern and northern populations.

Life histories are an accounting of the major components of fitness: survivorship and fecundity through time. The life histories of organisms represent a compromise or tradeoff among the maximization of survivorship and fecundity and the minimization of time. These tradeoffs are usually interpreted as adaptations to the historical and ecological context in which the organisms have evolved and currently live (Stearns, 1976, 1992; Roff, 1992, 2002). Life-history theories are unifying concepts, usually invoked to explain, and more rarely to predict, how fitness traits will sort out individually or group into “syndromes” (Dingle, 1986; Buoro and Carlson, 2014) along a continuum between extremes. Life-history theory predicts how selection should shape the evolution of fitness traits and, therefore, should be tested by genetic differences in fitness traits along a putative selection gradient. When comparing contemporary populations, differences in fitness traits among them are presumed to be adaptive and, therefore, the direct product of selection in the environmental context in which they exist. Herein, we compare fitness traits of *Wyeomyia smithii* among populations that differ in their propensity to bite over a geographic gradient.

Following the evaluation of fitness and the life history traits that define it, we explore the genetic architecture underlying differences between biting and non-biting populations. These architectures are formed by composite genetic effects ranging from simple additivity, wherein substituting a single allele for another has a fixed and independent effect, to the more complex actions of dominance, maternal effects, and different orders of epistasis. These various genetic effects have all been demonstrated to play a role in the evolution of populations; however, the emphasis has centered on the ways in which additive genetic effects shape the evolutionary process, and this is especially true in the realm of population genetics (Hansen, 2013). Additive effects have long been thought to be the primary vehicle upon which selection operates (Lande, 1980; Lande and Arnold, 1983; Lewinton, 1978), and indeed additive effects remain the central pillar solidifying our understanding of selection and the evolutionary process. While not as pervasive, dominance has played an important role in various phenomena that impact how

populations will change. It is thought to underlie primarily the negative effects imposed on populations by inbreeding depression (Willis, 1999; Lande and Schemske, 1985; Dudash and Carr, 1998; Simmons and Crow, 1977), as well as the positive effects occasionally observed in offspring of distantly related parents (hybrid vigor) (Lynch, 1991). Epistasis has more recently been elevated from an afterthought or an unfortunate complication in mathematical models to an important factor that has played a significant role in shaping the genetic architectures of species during the evolutionary process (Fenster and Galloway, 2000; Fenster and Dudash, 1994; Carter *et al.*, 2005; Demuth and Wade, 2005). Because additive, dominance, and epistatic effects have all played a role in the evolutionary process, and because we have no *a priori* reason to definitively rule any of these effects out, we used line cross analysis to reveal the genetic architecture involved in the transition from a biting to a non-biting phenotype in populations of the pitcher plant-mosquito *Wyeomyia smithii*.

Methods

Fitness Assay and Life History

Populations used to estimate fitness and which underwent subsequent life history analysis consisted of cohorts of 980 newly emerged larvae collected on a single day (all cohorts emerged from eggs over less than a 2 day period) and reared in the same manner described in the “Fitness Assay” section in the second chapter. Populations included two representatives from the northern clade (KC and GM) and two from the southern clade (WI and LI) the former of which do not bite and the latter of which do (see Table S1 for details).

Analyses

Pre-adult development time and total lifespan were calculated as days from hatch to adult eclosion or adult female death, respectively. Adult female longevity was calculated as the average days to female death minus average days to female eclosion. Degree of iteroparity was calculated as the standard deviation in week of hatch emergence. Cohort

replacement rate (R_0) was calculated as total offspring hatch/980, the number of hatch in the parental generation. In lieu of age at first reproduction, which would include only one or a few individuals of a large cohort, we calculated the age at which an experimental cohort replaced itself, i.e., $R_0 = 1.00$. As a composite index of fitness incorporating age-specific survivorship and fecundity, we used Laughlin's (1965) capacity for increase:

$$r_c = \ln(R_0)/T,$$

where $T = \sum xH_x / \sum H_x$ and H_x = number of offspring hatch counted at age x .

Geographical variation in development time, adult longevity, fecundity, iteroparity, R_0 , age at cohort replacement and r_c was evaluated using two-way ANOVA with replication, with geography and host availability as treatments. In all cases, geography, and presence/absence of the host, were analyzed as fixed effects. ANOVAs were run using Excel 2007.

Line Crosses Analysis

Two distinct line cross experiments were performed both between a northern and southern population. In the first experiment we used a population originating from Maine (KC) and crossed it to a population originating from Florida (WI); the second experiment consisted of a population from Newfoundland (GM) crossed to a population from Alabama (LI). In both cases the crossing design is consistent and can be seen in Figure S2. Note that northern populations do not bite and as such no variance estimate can be derived; however, the effect of the northern parent can be estimated using backcross generations. All other rearing protocols and analyses are consistent with the line cross performed in Chapter II.

Results

Fitness Assay and Life History

As in the cages in which biting was observed, hosts were observed continuously in the 0% biting populations, where no female was seen with a kinked labium or with blood in her abdomen. In cages where southern females ingested blood, dark red-black splatters from excreted hemoglobin were routinely observed on the absorbent white cage floor; no such splatters were ever observed in the cages with 0% observed biting. These results support earlier reports that no females from populations at latitudes $\geq 38^\circ\text{N}$ or altitudes $\geq 600\text{m}$ have ever been observed to bite (Smith, 1902; Smith and Brust, 1971; Istock *et al.*, 1975; O'Meara *et al.*, 1981; O'Meara and Lounibos, 1981).

Southern populations maintained a persistent and significantly lower capacity for increase (Figure 3.1) irrespective of whether or not they had access to a blood source relative to northern populations that never blood-feed.

Regardless of host availability, southern females did not differ from northern females in pre-adult development time, nor in degree of iteroparity (Figure 3.2, left and Figure 3.3 left, respectively). Southern females had lower per-capita fecundity than northern females, despite significantly greater adult female longevity (Figure 3.3 right, Figure 3.2, right, respectively). The increased fecundity of northern populations resulted in a higher cohort replacement rate (Figure 3.4, left) with a non-significant earlier age at cohort replacement (Figure 3.4, right). Geography accounted for 67% and 50% of the variation in cohort replacement rate and non-significant variation in age at cohort replacement, respectively, so that we are neither confident in rejecting an effect of geography nor accepting the null hypothesis of no effect of geography

Line Crosses Analysis

Two replicate comparisons (see Figure S3 for crossing design) between southern biting populations and northern non-biting populations reveal that additive genetic effects play an important role in determining the differences between populations for this phenotype (Figure 3.5). However, in neither replicate cross are additive effects alone sufficient to explain the difference between southern biting and northern non-biting populations ($\chi^2(6) = 38.009, P < 0.0001, \chi^2(6) = 34.893, P < 0.0001$, Figure 3.5 upper and lower, respectively). Incorporating dominance as well as maternal effects increases the goodness

of fit between the observed generation means and the expected. However, in neither cases are we able to accept the ADM model to explain the observed means and variances ($\chi^2 (3) = 10.279, P = 0.0163, \chi^2 (3) = 13.551, P = 0.0036$, Figure 3.5 upper and lower, respectively). Rejection of the most complex model available (additive-dominance-maternal) leads us to invoke epistasis as likely being involved in the difference between southern and northern populations at the biting phenotype. The direction of dominance is interestingly not stable across comparisons: in the cross between Florida and Maine (Figure 3.5 upper), the blood-feeding phenotype is at least partially dominant to the non-biting phenotype whereas the reciprocal is true for the comparison between Alabama and Newfoundland (Figure 3.5 lower).

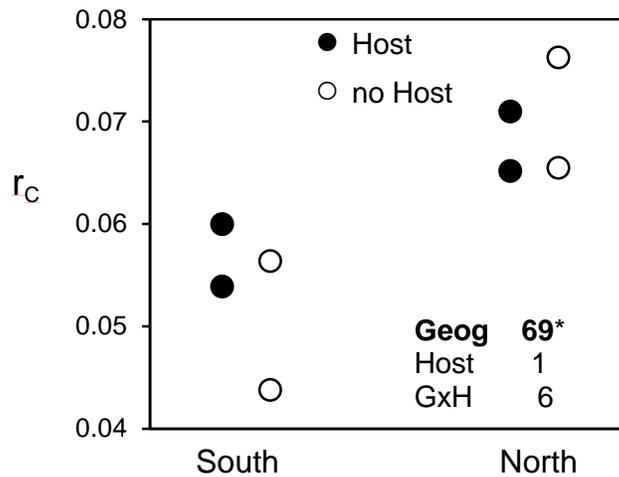


Figure 3.1 Capacity for increase measured in biting southern and non-biting northern populations. Inset gives the total sum of squares explained by geography (Geog), host availability (Host), and the interaction of the two factors (GxH). Factors bolded are significant at the $P = 0.05$ threshold for significance, $*P < 0.05$.

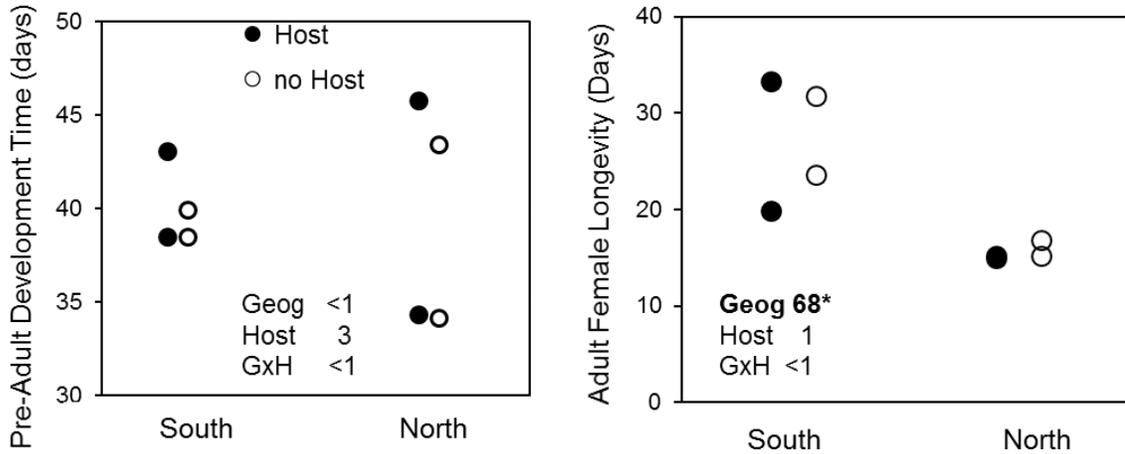


Figure 3.2 Female pre-adult longevity (left) and adult longevity (right). Inset gives the total sum of squares explained by geography (Geog), host availability (Host), and the interaction of the two factors (GxH). Factors bolded are significant at the $P = 0.05$ threshold for significance, * $P < 0.05$, ** $P < 0.01$

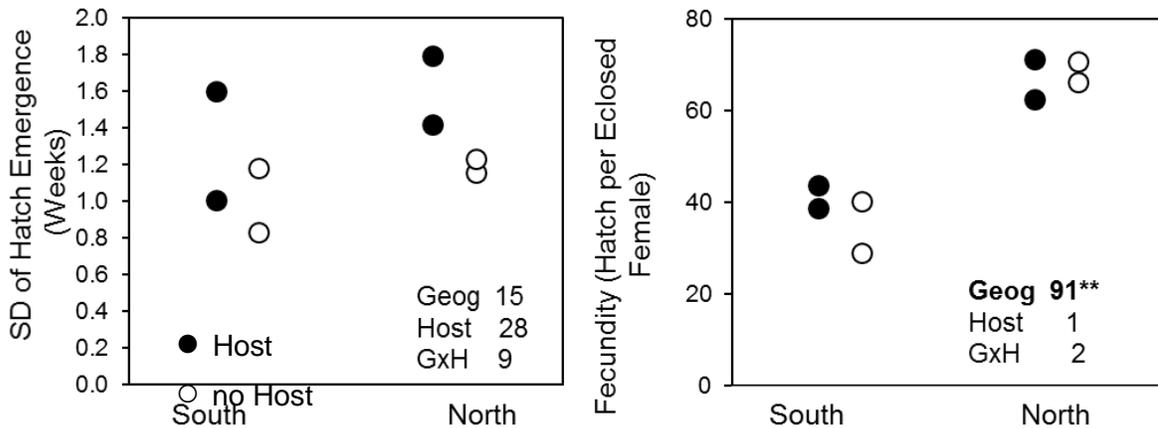


Figure 3.3 Female iteroparity expressed as the standard deviation (SD) in week of hatch emergence (left) and fecundity (right). Inset gives the total sum of squares explained by geography (Geog), host availability (Host), and the interaction of the two factors (GxH). Factors bolded are significant at the $P = 0.05$ threshold for significance, * $P < 0.05$, ** $P < 0.01$

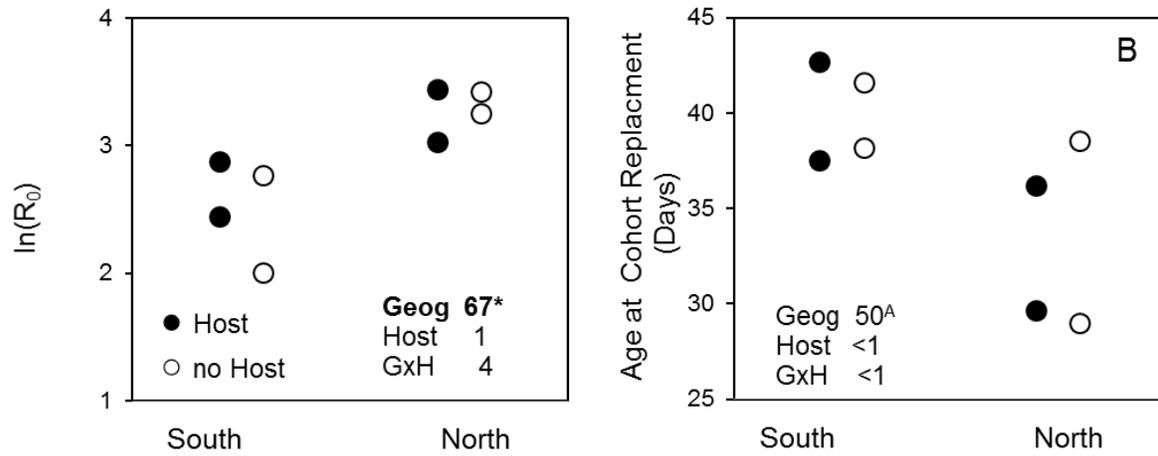


Figure 3.4 Cohort replacement rate (left) and age at cohort replacement (right). Inset gives the total sum of squares explained geography (Geog), host availability (Host) and the interaction of the two factors (GxH). Factors in bold are significant at the $P = 0.05$ threshold for significance, $*P < 0.05$, $^A P = 0.112$.

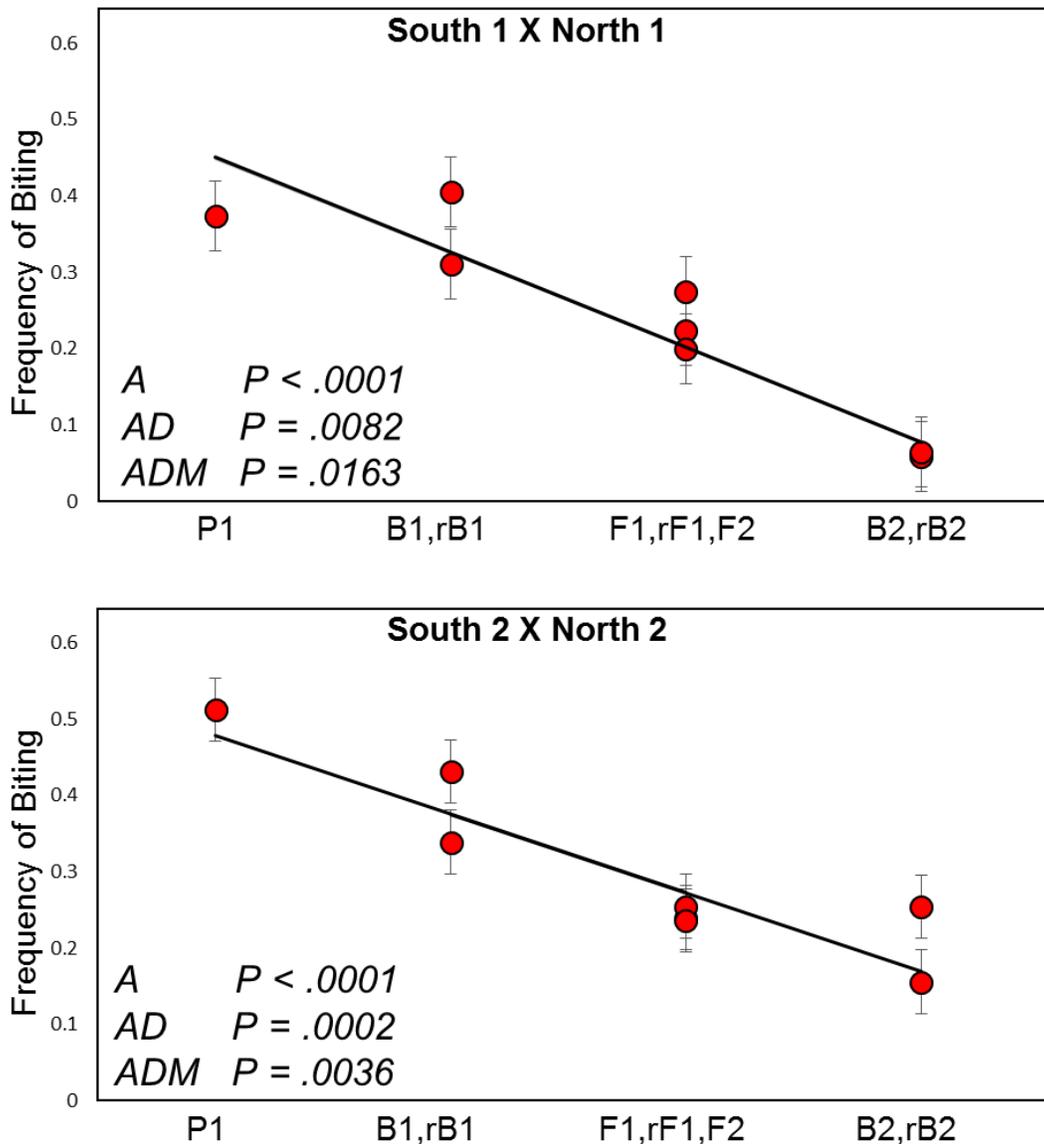


Figure 3.5 Results of two line crosses contrasting southern biting populations to northern non-biting populations. Upper panel shows Florida versus Maine, lower panel shows Alabama versus Newfoundland. X axis labels are generations of varying genetic ancestry due to crossing design and in order are; the southern parent, the backcross and reciprocal backcross to the southern parent, the F1 offspring between the southern parent and the northern parent, the reciprocal sexes relative to the former cross, the F2 offspring, and the backcross and reciprocal backcross to the northern population. The Y axis gives frequency of female biters in each of the crosses (denote by red dots). Solid line represents the additive expectation. Insets show the goodness of fit to simple additive (*A*) additive/dominance (*AD*), and additive/dominance/maternal (*ADM*). P-values below .05 demonstrate a significant departure from the model expectations and thus a rejection of that model as adequate to explain genetic differences between populations.

Discussion

Response to selection shown in Chapter 2 and geographic variation in propensity to bite (Table S1) demonstrate that blood-feeding is a highly heritable trait and has undergone evolution during the northward dispersal of *Wyeomyia smithii* in North America. Northern non-biting populations (Table S1) achieve greater lifetime female fecundity, greater lifetime cohort replacement rates (R_0), greater iteroparity, and greater capacity for increase (r_c) than southern biting populations, regardless of the presence or absence of a vertebrate host (Figures 3.1-3.3). At the same time (Figure 3.4), northern females do not differ from southern females in age at cohort replacement ($R_0 \geq 1.0$) though the non-significant trend is towards northern populations achieving cohort replacement at a younger age than southern populations. The only trait in which southern populations show a distinct advantage is adult longevity (Figure 3.2). These results show that there has likely been no demonstrable cost to fitness in the evolutionary transformation from a blood-feeding to an obligately non-biting mosquito. A low propensity to bite among mosquitoes in general has been correlated with relatively nutrient-rich and predator-free larval habitats and to a limited availability of vertebrate hosts for adults (Corbet, 1964, 1967; Lounibos *et al.*, 1982; O'Meara, 1985b; Clements, 1992). Even viciously biting, invasive species such as *Aedes aegypti* and *A. albopictus* include females that may produce some fertile eggs without a blood meal under conditions of low larval density and high larval nutrition (Mori *et al.*, 2008; Ariani *et al.*, 2015). However, in all cases, whether it be vicious or low-level biting, there is both an extrinsic and intrinsic cost to blood-feeding due to finding and consuming a blood meal. Blood-feeding mosquitoes must locate a vertebrate host and survive the taking of a blood meal (Edman and Scott, 1987; Darbro and Harrington, 2007; de Silva *et al.*, 2014). In addition, blood-feeding does not constitute a free meal. The breakdown of hemoglobin, especially heme, imposes potentially lethal oxidative stress, invoking both biochemical and morphological detoxification mechanisms (Pascoa *et al.*, 2002; Graça-Souza *et al.*, 2006; Esquivel *et al.*, 2014; Nikbakhtzadeh *et al.*, 2016). Herein, consideration of *W. smithii*, with its consistency of habitat over more than 30° of latitude (Bradshaw and Lounibos, 1977;

Bradshaw, 1983; Bradshaw and Holzapfel, 1986; Buckley *et al.*, 2003) and its transition from a biting habit in the south to an obligately non-biting habit in the north, has led us to conclude that blood-feeding itself has not been driving life-history evolution in this species. Importantly, at the same time, it is clear from the present study that the evolutionary transition in *W. smithii* from a blood-feeding to an obligately non-biting lifestyle has resulted in an increase, not a decrease, in most measures of reproductive success and, indeed, an increase in overall fitness itself.

Understanding the fitness consequences of the transition from biting to non-biting lends insight into why the contrasting phenotypes are maintained, but this does little to inform how this medically-important trait is maintained at the genetic level. To better appreciate and articulate the latter point we conducted two line crosses using replicate northern and southern populations. Research aimed at understanding the basis of biting has been extensive in effort and broad in methodology, including studies examining physiology (reviewed extensively in Clements, 1992) and olfaction (Hallem *et al.*, 2004; Takken, 1996; Liu *et al.*, 2010), as well as genetic expression (Rinker *et al.*, 2013; Bonizzoni, 2011; Sanders *et al.*, 2003; Dana *et al.*, 2005; Das *et al.*, 2010). Despite the wide methodological net being cast, most study systems are constrained by the fact that a fully comparative approach is not possible given that within a species of mosquito, all female individuals either bite (*e.g. Anopheles gambiae*), or do not (*e.g. genus Toxorhynchites*). Comparisons between biting and non-biting species are impractical as the differences that exist between species having nothing to do with biting are too numerous, and techniques which require crossing are impossible. The optimal study system for a comparative approach would involve either closely related sub-species that differed in a phenotype of interest or a single species wherein different populations are phenotypically variable. Both of these approaches have been successfully used to characterize the genetic basis and the genetic architecture of evolutionarily relevant traits in a number of diverse taxa (Streisfeld, 2013; Hohenlohe, 2010; Coyne, 1989, 1993). The *Wyeomyia smithii* system allows for such a comparison to be made using crosses between biting and non-biting individuals of the same species in which northern populations have transitioned to non-biting through evolutionary time. In both crosses, the most complex model involving

additive, dominance, and maternal effects was unable to explain the observed variation in blood-feeding propensity between generations of varying genetic ancestry (Figure 3.5). As such, epistasis is likely a component of the genetic architecture underlying the transition to non-biting. Epistasis as a necessary factor to explain the difference makes sense given the distance and time that separates northern and southern populations. Previous work using line cross analysis in the *W. smithii* system has indicated that non-additive effects play an important role in determining the differences between populations (Lair *et al.*, 1997), and that the likelihood of seeing more complex forms of non-additive effects (epistasis) increases as the time between divergence of crossed populations increases. This trend of epistasis dictating differences between distantly related individuals, while additive and dominance effects predominate between close relatives, is supported by studies demonstrating that outcrossing depression is generally due to the breakup of favorable gene-gene interactions (Carson and Templeton, 1984; Edmands, 1999; Demuth and Wade, 2007) while inbreeding depression is often due to the within-locus effects of dominance (Willis, 1999; Simmons and Crow, 1977). While epistasis is necessary to invoke in both north-south crosses, additive and dominance effects remain important factors in both cases. Of particular interest is the direction of dominance, which is not stable between crosses. In the cross between Maine and Florida, the southern biting phenotype is at least partially dominant to the northern non-biting phenotype, however the reciprocal is true in the cross between Newfoundland and Alabama. The opposing sign of dominance in replicate comparisons suggests that a transition to a non-biting phenotype may not be a narrow alley, but instead an intersection in which populations may take one of many directions during the transition to non-biting. The current study lays the groundwork for future research that seeks to characterize the genetic basis of the transition to non-biting.

Bridge: From genetic architecture to genes.

The previous chapters have quantitatively evaluated the consequences of the biting phenotype and its loss in terms of fitness, life histories, and genetic architectures for both a line artificially selected for biting and naturally varying populations. The final chapter of this thesis moves down one further level of biological integration, to the level of the gene, and uses both artificially and naturally variable populations to ask what gene/genes are consequential in the transition to a non-biting life style. The specific goal of this study is to characterize the genes potentially responsible for the shift from a biting to a non-biting life style. To do this we use RNA-sequencing to evaluate the expressional profiles of various biting and non-biting lines and treatments, followed by a statistical comparison highlighting those genes that are significantly differentially expressed between treatments. Moving down to the level of the gene is a natural extension of the previous experiments but also serves as a first and important step in developing a way to interrupt a phenotype that kills millions of people each year.

CHAPTER IV

DIFFERENTIAL GENE EXPRESSION AS A CONSEQUENCE OF THE TRANSITION TO NON-BITING IN *WYEOMYIA SMITHII*

Rudyard J. Borowczak, Alida T. Gerritsen, William E. Bradshaw, and Christina M. Holzapfel, conceived of the work and designed the experiments presented in this chapter. R.J.B. performed the experiments, and Doug Turnbull executed the molecular aspects of this work. R.J.B., W.E.B., C.M.H., Allison Fuiten, and Ryan Meng performed the analyses. R.J.B., W.E.B., C.M.H., and A.F. and R.M. wrote the paper.

Introduction

Arthropod hematophagy, or blood-feeding, exerts health and economic costs on humans and other animals that are difficult to overstate. Malaria alone kills over 400,000 people a year and afflicts nearly three orders of magnitude more than the number of mortalities (World Health Organization, 2016). Many diseases afflicting humans and vectored by insects emerged long ago and have likely had a long and complicated evolutionary history with humans, such as the Plasmodia responsible for malaria (Loy et al., 2017; Carter and Mendis, 2002), while others such as Zika and Dengue viruses have only recently have become major health risks (Holmes and Twiddy, 2003). Vector-borne diseases are diverse, encompassing both prokaryotes and eukaryotes, ranging from viruses (e.g. Dengue and Yellow fever) and protists (e.g. malaria) to nematodes (e.g. filariasis).

Without the blood-feeding habit of many insects, vector-borne diseases would have no obvious mode of transmission, and so while the bite itself is not harmful or lethal, it is the common pathway of all vector-borne diseases. Mosquitoes (Diptera: Culicidae) are the primary vectors of many of the most impactful human diseases and are themselves an extremely diverse group comprising approximately 3,500 identified species (Harbach, 2017). Female mosquitoes seek out a blood meal from a vertebrate host and use the lipid- and protein-rich blood for ovarian development (Clements, 1992). However, the degree

to which female reproductive success is linked to blood-feeding varies across species, and can be thought of as a continuum: at one extreme females are totally dependent on blood to yolk their eggs and produce offspring; this tactic is termed anautogeny. Examples include most representatives from the genus *Anopheles* such as *gambiae*, the major vector of malaria on the African continent (Clements, 1999). At the other extreme are some species and three entire genera (*Malaya*, *Toxorhynchites*, and *Topomyia*) that never bite. These mosquitoes generally acquire the protein and lipid necessary for reproduction prior to the adult stage, avoiding the risk and energy expenditure associated with host-seeking and blood-feeding (Downes, 1958; Foster, 1995; Rattanakul et al., 2007; Wahid et al., 2007; Miyagi et al., 2014; Zhou et al., 2014). Between these extremes lie a number of reproductive strategies in which blood-feeding is optional, but successfully finding a host generally leads to higher reproductive success (Corbett, 1967). The cycle of disease transmission for those pathogens that are not able to pass directly from mother to offspring (vertical transmission) requires a vector that sequentially bites an infected host and then bites one or more uninfected hosts (horizontal transmission). For many mosquitoes, especially anautogenous varieties, multiple blood meals often improve reproductive success (Briegleb, 1993; Corbett, 1967), imposing a selective pressure to bite more than once, with a correlated and positive effect on vectorial capacity (Garrett-Jones, 1964). Because the common thread that binds all vector-borne diseases together is the biting behavior of the vector, an efficient way of combating disease transmission is to approach the problem at the level of the bite.

Wyeomyia smithii is unique among all known species of mosquitoes in that in one part of its range, populations are willing biters, while in the rest of its range populations have transitioned to an obligately non-biting lifestyle (Smith and Brust, 1971; Bradshaw, 1980; O'Meara et al., 1981; Bradshaw and Holzapfel, 1983). This evolutionary transition has a genetic basis since selecting on blood-feeding in a population with a low propensity to bite dramatically increases the rate of biting (see Chapter II). The natural and heritable variation in biting propensity among fully interfertile populations of a single species offers a unique opportunity to determine the genetic basis of biting in mosquitoes.

Research devoted to understanding the genetic basis of biting is sizable and diverse, using genomic, proteomic, and transcriptomic approaches across a wide variety of mosquito taxa (Holt, 2002; Ribeiro, 2003; Bonizzoni *et al.*, 2010; Phattanawiboon, 2016). However, in all cases, researchers are limited by systems in which, given the opportunity, their mosquito of interest will take a blood meal. *Wyeomyia smithii* presents no such constraints, providing researchers with the opportunity to understand how this natural transition from biting to obligate non-biting has occurred. Herein we use RNA-sequencing to determine the expression profiles of genetically programmed biting and non-biting individuals in *W. smithii*. To refine our search to genes most likely to be causal or consequential to the biting phenotype itself, we use a design that incorporates both the natural variation in blood-feeding and a line selected for biting. First, we compare biters from the line selected for enhanced biting frequency (OB) to non-biters from its weakly biting progenitor population from the state of Florida (WI), followed by a comparison again using biters from the selected line and northern non-biting individuals from a population in the state of Maine (KC) (see Figure S1). We then consider only those genes that are differentially expressed in the same direction in both comparisons as those most likely to underlie the tendency to bite versus the tendency to not bite, after which we map the genes identified by this design onto biological pathways in order to gain an understanding of known gene functions.

Methods

Rearing

Larval mosquitoes were reared in CT rooms programmed to run a sinusoidal daily temperature fluctuation with a maximum temperature of 35°C and a minimum temperature of 15°C, relative humidity at 80%, and the light regimen was set to a 18:6 LD cycle. This regimen promotes continuous, non-diapause development for all populations used in this experiment. Larvae were fed *ad lib.* a 4:1 mixture by volume of ground freeze-dried brine shrimp (San Francisco Bay Brand, 8239 Enterprise Drive, Newark, CA 94560, USA) and guinea pig chow (Geisler Guinea Pig Chow, Sergeant's

Pet Care Products, 10077 S 134th St, Omaha, NE 68138, USA) once a week. Adults were offered a continuous supply of organic (pesticide free) raisins as a carbohydrate source. Adult cages were constructed from 1 5L food-safe buckets. For all populations larvae were kept as 35 per dish until they developed into pupae, at which time they were removed to an adult cage, which was furnished with a large leaf of the pitcher plant *Sarracenia purpurea* for oviposition.

Sampling

Experiments consisted of four replicates of three treatments. The first treatment used obligate non-biters from a northern Maine population; the second treatment used disinterested biters from the outbred Florida population from which the selected line was established; and the third treatment sampled biters from the selected line. Prior to sampling in the second treatment, a large cohort of adults emerging over a three-day period were maintained in an adult cage and offered a rat for 15 min three times a week for two weeks and biting females were removed. The remaining females were scored as disinterested biters. All samples were taken over a 15 min interval, between 1200-1400 h subjective mosquito time, with an anaesthetized host (*Rattus norvegicus*) present in the cage. In the first two treatments, frozen heads were paired with their respective abdomens, sex was determined from the separated abdomens, and only the corresponding female heads were retained as samples. In the third treatment, only females bite; consequently, heads of biting females were retained without sexing the abdomens. To minimize the imbibition of blood, females were scored as biters when they had inserted their fascicle to the point where their labium was distinctly kinked.

Adults were aspirated from the cage and immediately flash frozen on a sterile Petri dish nested in dry ice under a dissecting microscope. Heads were then excised and homogenized in Trizol Reagent in microfuge tubes likewise nested in dry ice. After homogenization, samples were maintained at -80°C until RNA extraction and cDNA synthesis.

RNA library prep and sequencing

RNA was extracted using the Zymo Direct-zol MiniPrep kit according to the manufacturer's protocol with the following modifications: Mosquito heads were homogenized in 600 μ l of Trizol reagent and centrifuged for 5 min at 6000 x g to pellet cuticle and debris. Following centrifugation, the cleared Trizol supernatant was used as input for the Zymo kit and the manufacturer's protocol for RNA purification was followed. RNA-seq libraries were prepped for Illumina sequencing using the Kapa Biosystems Stranded mRNA-seq kit (KK8420) using a normalized quantity of 720 ng of total RNA for each sample as input. Prior to sequencing on the Illumina HiSeq 4000, we determined library fragment size distributions using an Advanced Analytical Fragment Analyzer, and measured final library concentrations by qPCR. An equal-molar amount of each library was pooled, and the final pool was sequenced in a single HiSeq 4000 flowcell lane, yielding ~27 million reads for each sample.

De novo transcriptome

We removed low-quality from RNA-seq reads using *process_shortreads* from *Stacks*, version 1.21 (Catchen *et al.*, 2013), and performed rare k-mer filtering and digital normalization using *kmer_filter* (to 20 read depth) from *Stacks*. We then generated multiple de novo transcriptome assemblies from the cleaned, filtered RNA-seq data using *Trinity*, Release 2013-11-10 (Grabherr *et al.*, 2011) set at different coverage cutoffs, varying from 1 to 7. For our final transcriptome assembly, we chose to proceed with the assembly generated by *Trinity* with the *min_kmer_cov* set to 5. We used the software packages *cegma*, version 2.4, (Genis *et al.*, 2007) and the accessory script *stats_assemble* within the software program *khmer*, version 1 (Crusoe *et al.*, 2015) to assess quality and completeness of our *de novo* transcriptome assemblies.

Differential Expression

We filtered the *de novo* transcriptome so that it only contained the longest open reading frame (ORF) per isoform group. We aligned the processed reads from the 12 RNA-seq libraries to this longest ORF transcriptome assembly using *Bowtie*, version 2.2.9 (Langmead *et al.*, 2009). We counted the number of reads that mapped to each contig in the longest ORF transcriptome using *samtools*, Release 0.1.14 (Wysoker *et al.*, 2009).

The read counts were analyzed to assess differences in gene expression between populations using the *R/bioconductor package DESeq2, version 1.12.4* (Love *et al.*, 2014). Count data were fitted to a negative binomial general linear model and, to control for multiple comparisons, a Benjamini-Hochberg correction was performed, followed by a likelihood ratio test to identify significantly differentially expressed contigs. We compared OB to WI and OB to KC, with four replicates per population. We constrained our dataset to contigs that were differentially expressed in the same direction in both the OB to WI comparison and the OB to KC comparison.

We compared our transcriptome contigs against the NCBI non-redundant protein (NR) database using the BLASTx algorithm (with an E value threshold set to 10^{-5}) to assign putative genes to our transcriptome contigs (Altschul *et al.*, 1997; NCBI).

Pathways Analysis

To gain putative functional information, we translated these differentially expressed contigs into amino acid sequences using the Trinity accessory script, *transdecoder*. We assigned KEGG Orthology identifiers to these translated contigs and identified KEGG pathways where these contigs were present using *BlastKOALA* (Kanehisa *et al.*, 2016), specifying the *Wyeomyia smithii* taxonomy group identifier and the KEGG genes database *family_eukaryotes*.

Results

RNA extraction and cDNA synthesis yielded twelve 100 bp single-end read libraries which varied in read number from approximately 24,000,000 to 32,000,000 (Table 4.1). Quality filtering and trimming performed by the module *process_short_reads* within the program *Stacks* resulted in fewer than 1% of total reads being discarded or trimmed suggesting high quality RNA extraction and cDNA synthesis. *Wyeomyia smithii*'s genome is not yet sequenced. Hence, de novo transcriptome assemblies were generated using the program *Trinity*. Kmer coverage cutoffs were varied from 1 to 7, in iterations of 2, in order to optimize the quality of the assembled transcriptome. Metrics used to evaluate the quality of transcriptomes included the mean contig length, cegma score, and

proportion of reads from each library independently aligning back to the transcriptome after assembly. Although no single transcriptome maximized all metrics, the assembly using a kmer cutoff of 5 did well in all facets and was chosen as the assembly with which to progress (Table S.2). The final transcriptome consisted of 129,538 contigs, with a mean contig length, cegma score, and alignment rate of the processed reads of 1777 bp, 96.37%, and 84.89%, respectively. Following transcriptome selection, the longest open reading frame from each isoform group within the assembly was identified and subsequently retained. Other contigs in the same isoform group were discarded. This procedure prevented over estimation of the number of contigs differentially expressed due to reads aligning back to multiple contigs (a consequence of alternative splicing), yielding a final assembly containing 38,490 contigs. Counts of the processed reads generated by the program *samtools* were input into the program *DESeq2* which uses a negative binomial distribution to estimate differential expression. Two comparisons were made using *DESeq2*. The first contrasted southern females disinterested in biting with biters from the selected line; the second contrasted biters from the selected line with females from the northern obligately non-biting population. The number of differentially expressed contigs was 3,238 for the former comparison and 16,469 for the latter (Figures 4.1 and 4.2, respectively).

Library	Total reads	Discards	Trimmed	Total kept	% kept
KC 1	27,369,096	73,952	773	27,295,144	0.997298
KC 2	29,813,599	81,591	959	29,732,008	0.9972633
KC 3	32,563,141	85,496	1,105	32,477,645	0.9973745
KC 4	30,589,825	81,754	1,089	30,508,071	0.9973274
OB 1	28,478,122	74,723	646	28,403,399	0.9973761
OB 2	28,313,428	74,252	850	28,239,176	0.9973775
OB 3	25,900,384	74,913	668	25,825,471	0.9971076
OB 4	28,163,440	77,027	605	28,086,413	0.997265
WI 1	30,485,096	81,751	747	30,403,345	0.9973183
WI 2	28,218,443	75,752	678	28,142,691	0.9973155
WI 3	28,312,610	76,354	624	28,236,256	0.9973032
WI 4	23,792,558	54,084	703	23,738,474	0.9977269

Table 4.1. Summary of raw and processed reads from the 12 sequenced libraries used for transcriptome construction.

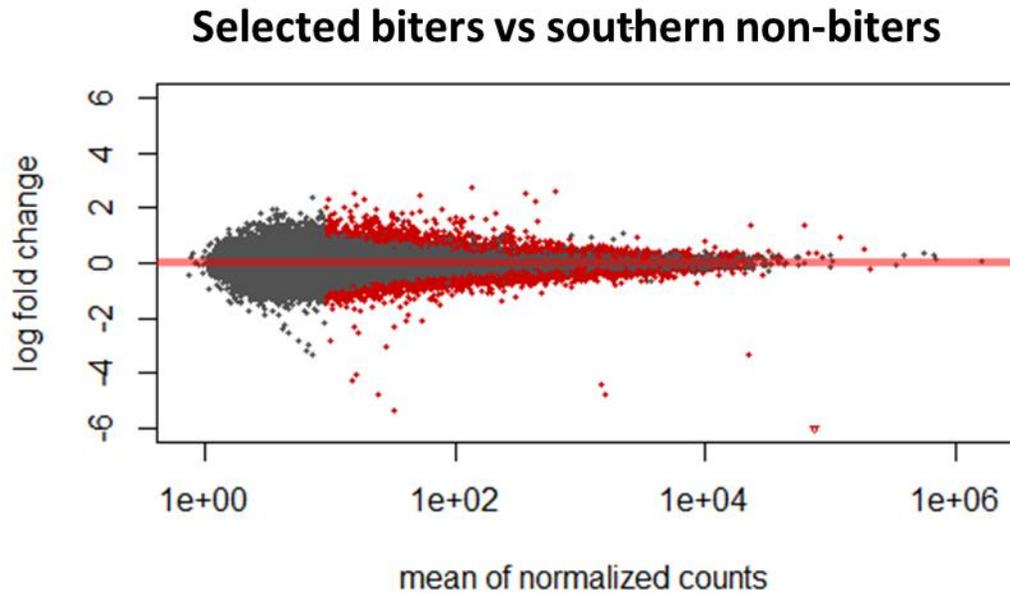


Figure 4.1. MA plot contrasting biters from the selected line with northern non-biters: MA plots show the log fold change in expression (y axis) against the degree of expression (x axis). Grey dots represent contigs that are not significantly differentially expressed between treatments, red dots represent contigs that are

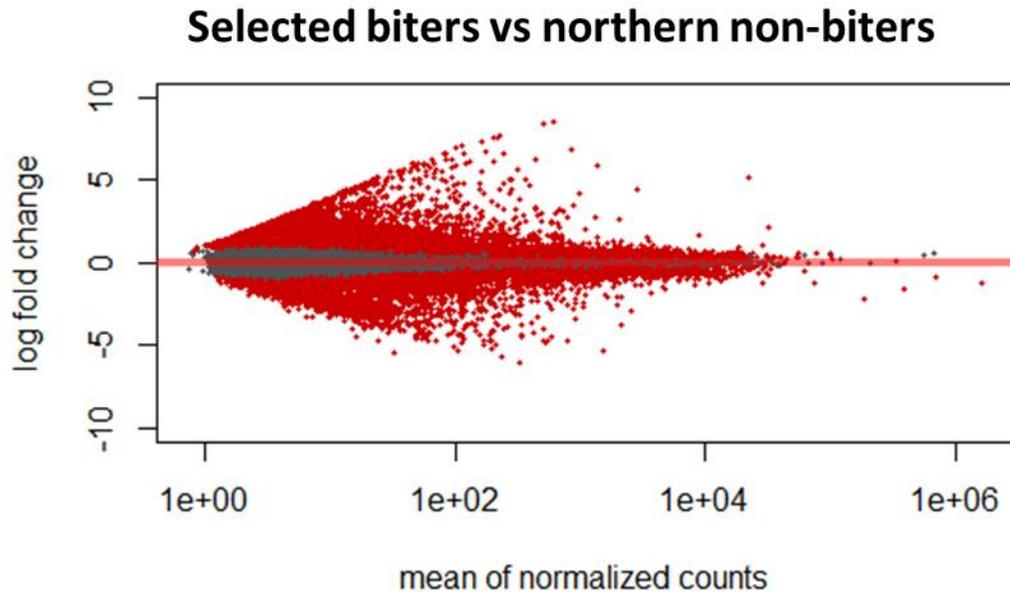


Figure 4.2. MA plot contrasting biters from the selected line with southern non-biters: MA plots show the log fold change in expression (y axis) against the degree of expression (x axis). Grey dots represent contigs that are not significantly differentially expressed between treatments, red dots represent contigs that are

The much larger degree of differential expression in the selected vs northern comparison relative to the selected vs southern was expected as the populations in the former comparison have been separated for approximately 200,000 years (Merz *et al.* 2013) while the latter differ only due to imposed selection. The contigs most likely to be causal or consequential in the transition from biting to non-biting are those that are differentially expressed in both of the above comparisons in the same direction. Refining the data set to meet this criterion yields 738 contigs downregulated in the selected line and 642 contigs upregulated in the selected line. To understand the biological function these 1,380 candidate contigs serve we submitted them to the KEGG database for annotation and pathway placement. In sum, 239 of our candidate contigs were assigned to unique genes in the KEGG database. Using the KEGG pathway mapper tool, we placed our annotated genes into known pathways; however, this approach was limited by incomplete annotation of invertebrate pathways. Therefore, we report only the pathways in which at

least one mosquito species has been mapped. The five pathways with the greatest number of differentially expressed genes (Figure 4.3) are: amino acid metabolism, oxidative phosphorylation, glycolysis, fatty acid degradation, and purine metabolism.

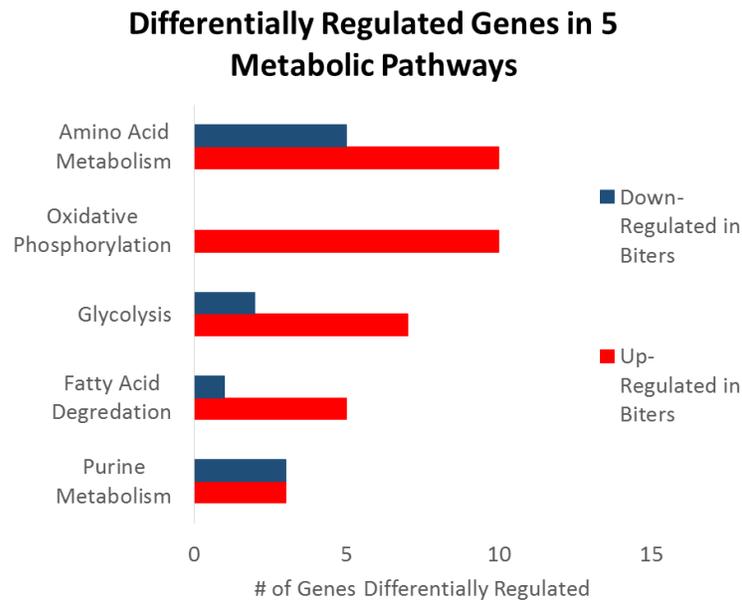


Figure 4.3. The pathways identified by KEGG. The greatest number of differentially regulated contigs originate from the 1,380 contigs differentially regulated in the same direction in both the selected-southern non-biting and selected-northern comparisons.

Discussion

Differential gene expression has been used to compare the genes involved before and after blood-feeding (Ribeiro, 2003; Bonizzoni *et al.*, 2010, Huang, 2015). However, *Wyeomyia smithii* allows for a comparison that is not possible in any other mosquito system: a regulational contrast between individuals of the same species in which one group is willing and capable of taking a take a blood-meal while the other is obligately non-biting. Natural geographic patterns in tendency to bite (Figure 2.1) and evidence from our previous work show a genetic basis of the transformation of a biter into a non-

biter. We need only to discover which genes have been responsible for that transition. In order to develop a list of candidate genes responsible for the maintenance of blood-feeding and candidate genes associated with obligate non-biting, we employed a pair of comparisons in which differentially expressed genes from one constrained the other. The first comparison contrasted biters from the selected line against non-biters from their progenitor population, which yielded 3,238 (Figure 4.2) differentially expressed genes representing 8.4% of the total transcriptome. The second comparison contrasted biters from the selected line (derived of a southern population) and non-biters from an obligately non-biting northern population and yielded a substantially greater number of differentially expressed genes (16,469, Figure 4.1) representing 42.8% of the total transcriptome. These results are in concordance with what we would expect given their distinct evolutionary histories: southern and northern populations have been separated for more than 200,000 years (Merz *et al.* 2013), while southern and selected individuals used in this experiment have been separated only by 19 generations of selection. Nevertheless, in both comparisons the individuals used differ in the phenotype of interest and thus are suitable for experimentation aimed at revealing the genes involved in blood-feeding and its loss.

We proposed that those genes differentially expressed in the same direction in both comparisons were those most likely to be involved in the evolutionary transition to obligate non-biting. Constraining our search in this way reduced our data set to 1,380 genes. We determined that these genes are disproportionately upregulated in the biters relative to the non-biters, and found primarily in metabolic pathways (Figure S4).

Our study system and experimental design are unique in two primary ways: first, we have the opportunity to compare biting individuals to non-biting individuals of the same species, whereas other systems necessarily compare blood-fed to non-blood-fed treatments; however, given the opportunity all individuals would blood-feed. Second, we use biting individuals at the time of the bite rather than after biting and compare these individuals to non-biters; this design omits genes that are consequential in processing an already achieved blood-meal. Our results contrast with past research aimed at elucidating the consequences of blood-feeding. Ribiero (2009) compared blood-fed and non-blood-

fed *Anopheles gambiae* and found a number of metabolic pathways differentially regulated that we also see in our analyses, including purine metabolism, glycolysis, and oxidative phosphorylation; however, in all cases but purine metabolism we find the opposite sign of differential regulation. Huang *et al.* (2015) used the Dengue fever vector *Aedes albopictus* to investigate the global transcriptional dynamics of both blood-feeding and induction of embryonic diapause and found, similar to our results, a significant upregulation of oxidative phosphorylation and amino acid degradation pathways in the blood-feeding treatments, however we did not find a number of genes important in explaining the differences between blood-fed and non-blood-fed treatments including various trypsins and vitellogenin.

In almost all cases, analyses comparing differential expression between blood-fed and non-blood-fed mosquitoes have shown an upregulation of the egg yolk protein vitellogenin in the blood-fed group (Ribeiro, 2003; Bonizzoni *et al.*, 2010; Huang, 2015). Vitellogenin is significantly upregulated in the northern obligately non-biting populations relative to the selected biters, but not in the selected biters relative to the non-biters in the unselected original population. How do we rectify this result that is so consistent across similar studies? First, northern *W. smithii* produce numerous clutches of eggs throughout their adult life without the input of blood. Thus, we would expect northern females to be constitutively expressing vitellogenin as they are continuously going through vitellogenesis.

In contrast, southern populations are facultatively autogenous, always producing a clutch of eggs before biting, but needing the input of blood to develop a second clutch. In this situation it is reasonable to suggest that since selected line females are biting, they have already produced their first clutch of eggs and would not then be expressing vitellogenin to any great degree. Furthermore, in most other studies investigating the expression profiles of biters and non-biters, the biting treatment has been allowed to feed to repletion, and tissue samples are generally taken well after this feeding event (Bonizzoni *et al.*, 2010; Ribeiro, 2003). In our case, we are interested in what regulational differences define biters at the time of the bite relative to obligate non-biters; as such, we are unlikely to detect many of the molecular cascades that occur in response to acquiring

a blood-meal. This design was intentional and we think the most likely to elucidate the genes that cause a biter to seek out a blood-meal as well as those genes that cause a non-biter to fail to.

The metabolic genes elucidated using RNA-SEQ are likely important in transforming a biter into a non-biter, however metabolic genes are unlikely to be causal in this shift but rather consequences of the transformation. Genes which cluster in endocrine, nervous system, and signaling pathways generally are better causal candidates, and have been identified during this study however; these pathways are poorly annotated for invertebrates within KEGG. Many of the genes annotated by a blast to the NCBI database are not found recognized by KEGG and are within the aforementioned pathways. Once pathway analysis programs become better annotated and gene clusters that are more likely to be causal than metabolic cascades are identified, then genomic tools such as knock downs, gene silencing, and gene editing can be used to assign a causal function of genes to the transition to non-biting. However, *W. smithii* does not currently have its genome sequenced, making some of these tools more difficult to use. Close relatives with their genomes sequenced and annotated e.g. *Aedes aegypti*, are the best options for evaluating the effects of gene manipulation.

In sum, the results of this study suggest that the maintenance of the biting phenotype incurs intrinsic costs, biting individuals anticipating a blood-meal upregulate numerous metabolic pathways relative to obligately non-biting northern and disinterested southern individuals. Previous studies have likewise suggested that blood is no free meal; the digestive pathways required for the breakdown of heme and protective measures required in order to tolerate subsequent oxidative stress are costly (Pascoa *et al.*, 2002; Graça-Souza *et al.*, 2006; Esquivel *et al.*, 2014; Nikbakhtzadeh *et al.*, 2016). Furthermore, extrinsic costs present themselves exclusively to biters, host searching can be energetically expensive and risky (Edman and Scott, 1987; Darbro and Harrington, 2007; de Silva *et al.*, 2014). The evolutionary history of the transition from biting to non-biting is likely a complex one, but the cost of maintaining the biting phenotype, in concert with the striking results in chapter III demonstrating a fitness loss in southern

biters relative to northern obligate non-biters present a novel rationale for why northern populations of *Wyeomyia smithii* have transitioned to an obligately non-biting lifestyle.

CHAPTER V

CONCLUSION

Organisms that maintain natural variation across ecogeographic gradients provide ideal natural endpoints of evolution and potential insight into the genetic mechanisms underlying evolutionary processes. In *Wyeomyia smithii*, we have such an organism that has evolved through space and time from the Gulf of Mexico to northern Canada while maintaining an unparalleled consistency of micro-habitat – the water-filled leaves of the carnivorous purple pitcher plant, *Sarracenia purpurea*. *Wyeomyia smithii* is the only species of mosquito that is blood-feeding in one part of its range and is an obligate non-biter throughout the rest of its range, producing repeated batches of eggs without resorting to blood-feeding. The primary focus of our research has been the pattern and processes involved in the evolutionary transformation from blood-feeding to obligate non-biting among fully inter-fertile populations of a single species of mosquito.

We first use *Wyeomyia smithii* to demonstrate that there is a geographic cline in propensity to bite and that this propensity to bite is a heritable trait. Selection for blood-feeding from a large out-bred population (14,000) collected in northern Florida resulted in more than double the incidence of biting after 10 generations of selection. Line crosses between the selected line and its weakly biting progenitor population revealed that response to selection involved the expected additive effects, but also maternal dominance but not maternal additive, dominance, or epistatic effects. The maternal dominance effect suggests a cytoplasmic or epigenetic effect on propensity to bite. Conventional wisdom predicts that blood-feeding and repeated batches of eggs should increase fitness. However, using a composite index of fitness incorporating survivorship, fecundity, and generation time, we observed a non-significant increase in fitness, but on the borderline of significance, so that we also cannot confidently reject the null hypothesis of no effect of increased blood-feeding on fitness.

When we examined the life-historical consequences of the evolutionary transition from a blood-feeding to an obligately non-biting lifestyle, clear patterns emerged. First, northern non-biting populations achieved higher fitness than southern biting populations, even in the presence of a vertebrate host and despite the greater adult female longevity in the southern biting populations. Rather, increased fitness in the northern populations resulted from double the lifetime fecundity of northern females. These results point to the novel conclusion that blood-feeding incurs both an extrinsic cost due to finding and feeding from a host, and an intrinsic cost in dealing with the potential physiological consequences of increased investment in protein degradation and stress imposed by increased osmolarity and iron toxicity resulting from the breakdown of hemoglobin. A blood-feeding lifestyle is a risky, expensive proposition. Avoiding these risks and costs permits re-allocation of resources to increased reproductive effort, greater per-capita female fecundity, a greater replacement rate per generation, and greater net fitness despite a shorter adult lifespan.

By comparing gene expression between southern females disinterested in biting and a selected line of vicious biters, we were able to identify gene expression specifically associated with selection on blood-feeding. We then were able to test for whether these same genes were involved in the evolutionary transition from blood-feeding to obligate non-biting by comparing gene expression between a northern, non-biting population and the vicious biting line. This approach enabled us to identify downregulation of amino acid metabolism, oxidative phosphorylation, glycolysis, and fatty acid metabolism in northern non-biting populations as compared with southern vicious biters as a result of selection accompanying the evolutionary transition from a blood-feeding to an obligately non-biting lifestyle. These results reflect a more active metabolism in preparation for blood-feeding than in maintaining reproduction with no anticipation of blood-feeding, underscoring our concept that blood-feeding is a costly enterprise.

The unique opportunity afforded by genetically determined but strikingly different lifestyles within a single interfertile species has enabled us to isolate gene expression specifically associated with the evolutionary transition from a blood-feeding to an obligately non-biting lifestyle. We are able to do so even though line-crosses between the southern and northern populations reveal a complex genetic architecture involving not only additive and maternal effects, but also dominance and epistasis. The signal that emerges from this complexity is that by reducing extrinsic and internal metabolic costs, northern, obligately non-biting populations have been able to out-reproduce blood-feeding, conspecific populations and, in so doing, achieve greater fitness.

Supplemental Material

Population*	State or Province	Latitude °N	Longitude °W	Elevation (m)	Number of ♀♀	Biting (%)
GM	Newfoundland	49.6	49.6	57.9	323	0.0
KC	Maine	46.2	68.3	365	264	0.0
NP	Maryland	38.3	75.5	18	456	0.0
DB	North Carolina	35.0	83.1	900	187	0.0
SF	North Carolina	35.1	82.7	670	282	0.0
SH	North Carolina	35.1	79.5	107	416	5.3
GS	North Carolina	34.2	78.3	20	407	4.6
CI	Alabama	31.2	88.3	152	404	16.6
BC	Florida	30.7	86.7	10	482	10.9
<i>LI</i>	Alabama	30.5	87.5	15	372	36.7
WI (Sel 0)	Florida	30.1	85.0	10	285	18.6
Sel 11					416	53.3
Sel 14					356	83.7

Table S.1. Origin and incidence of biting among populations of *Wyeomyia smithii*.

*Boldface indicates populations in the northern clade of *W. smithii* (Merz *et al.*, 2013). Population acronyms refer to populations in previous publications from this lab. Sel, number of generations of selection for vicious biting in the WI population.

Kmer Coverage Cutoff	Contig Number	Median Length	Mean Length	N50	CEGMA Complete	CEGMA Partial	Alignment Rate
1	256372	964	1775	3296	97.58	100	78.32
3	183184	1081	1923	3499	98.39	100	81.25
5	129538	987	1777	3186	96.37	100	84.89
7	93203	771	1337	2212	86.69	99.6	84.54

Table S2: Summary statistics for transcriptomes assembled

*Bold indicates transcriptome used for analyses

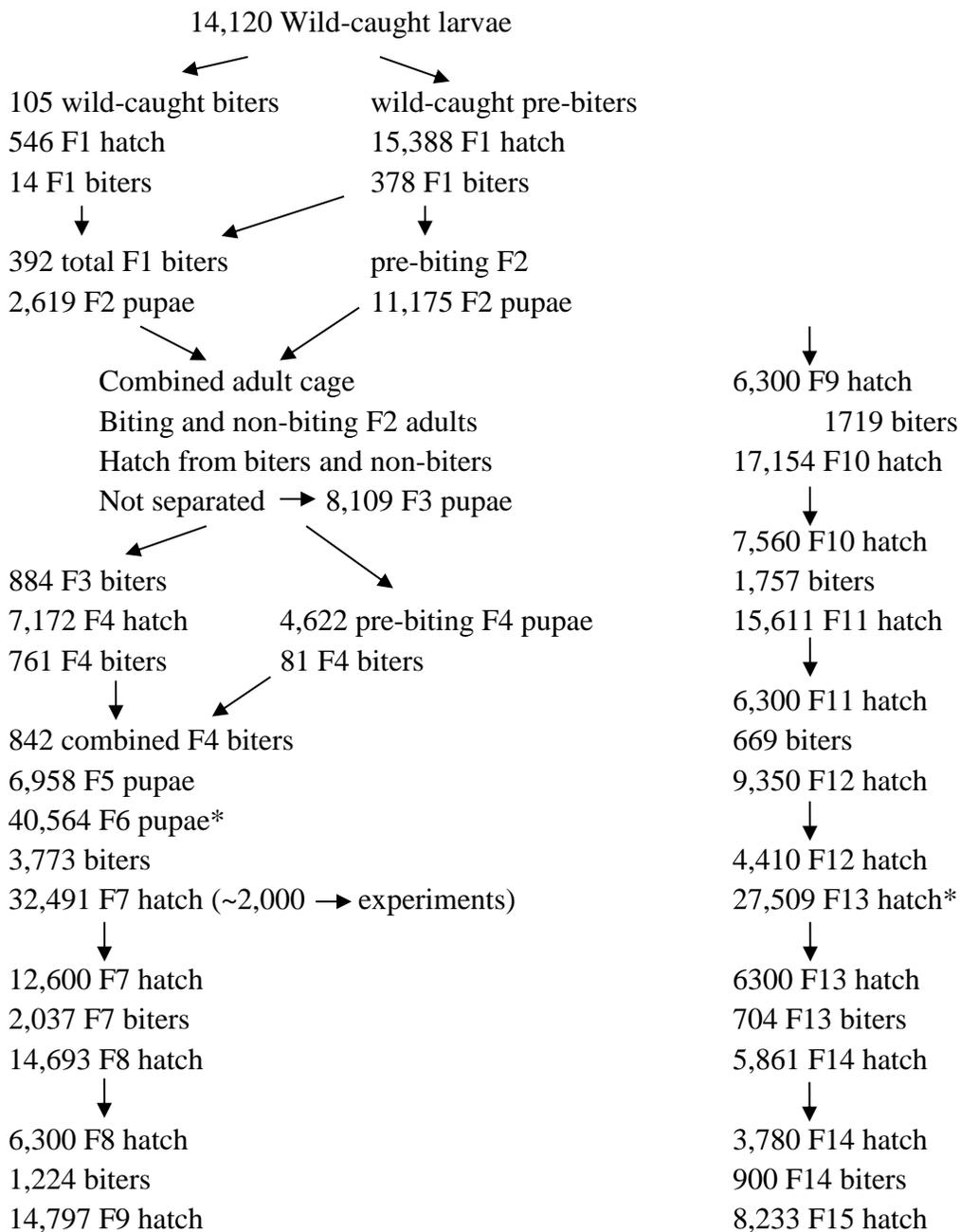


Figure S.1. Generation and maintenance of the line selected for avid biting. The complex manipulations in early generations are due to the fact that, initially, total hatch from biting females was not sufficient to generate a cohort replacement rate (R_0) greater than 1.0. Consequently, until $R_0 \geq 1.0$ was reached, hatch from biting females was comingled with hatch from females of the same generation that had not taken a blood meal (pre-biters). Until $R_0 \geq 1.0$, all hatch from blood feeding females were used to generate the selected line; thereafter, hatch in excess of $R_0 > 1.0$ were available for

experiments. * Reared through one generation without a blood meal to augment the selected line.

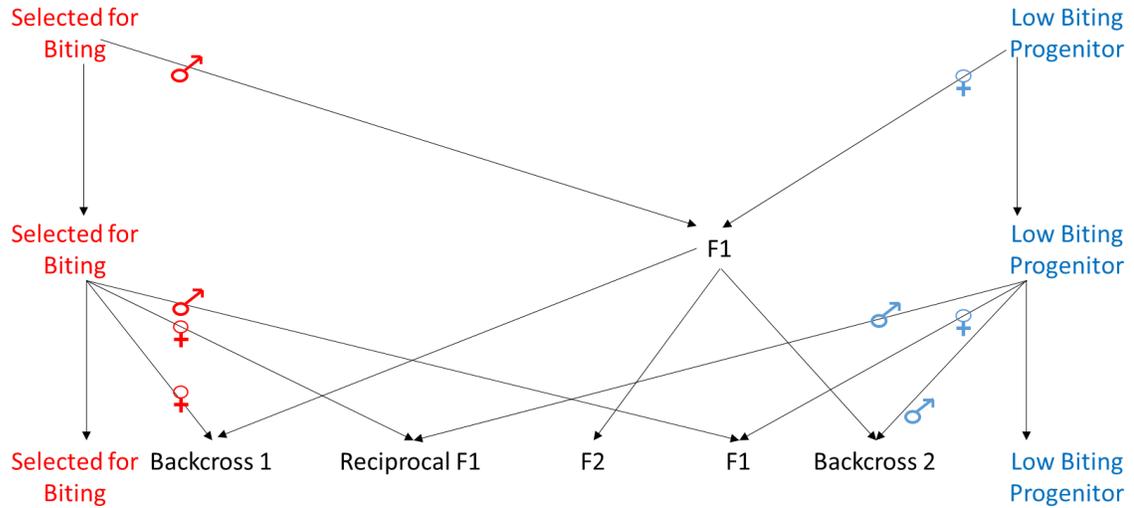


Figure S.2. Crossing design for the line selected for biting and its weakly biting progenitor.

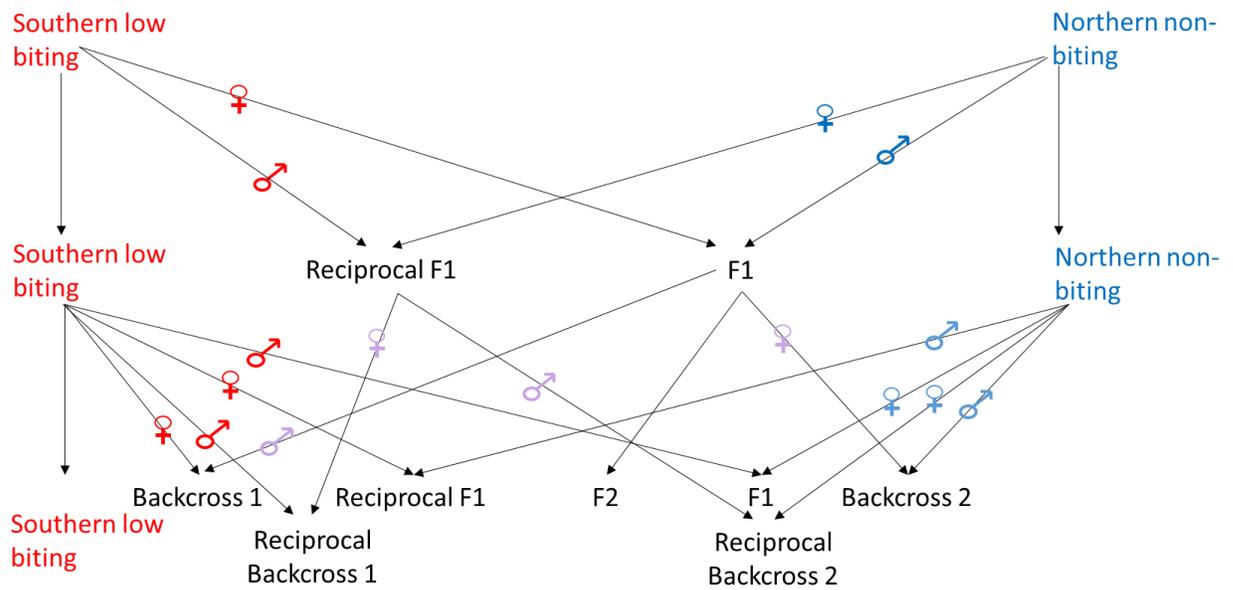
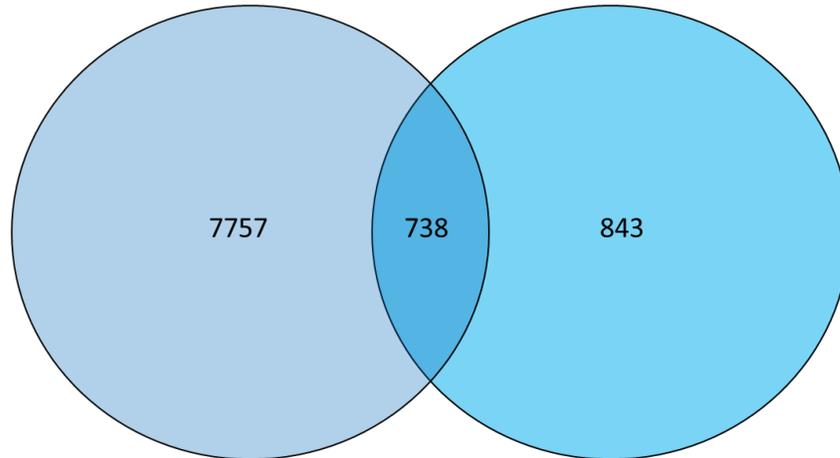


Figure S.3. Crossing design for replicate northern non-biting and southern biting populations.

Down-regulated Contigs in the Selected Biters

Selected vs North non-biting (down) Selected vs South non-biting (down)



Up-regulated Contigs in the Selected Biters

Selected vs North non-biting (up) Selected vs South non-biting (up)

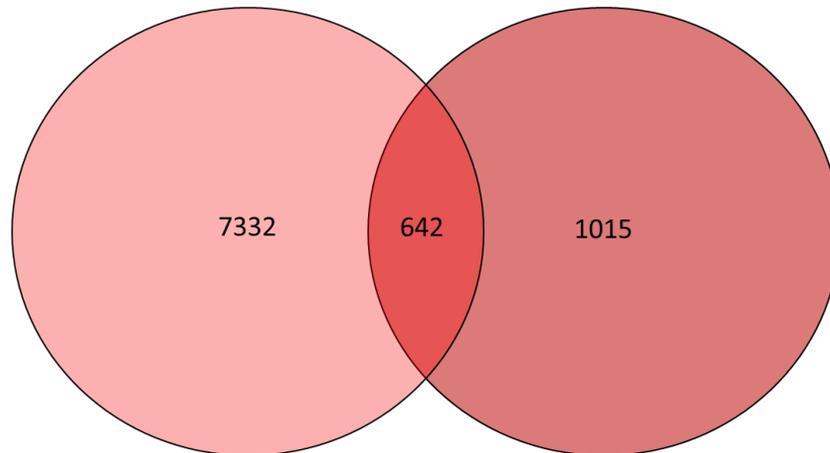


Figure S.4. Down-regulated contigs (upper) and up-regulated contigs (lower) in the selected vs northern non-biting and selected vs southern non-biting comparisons. Values in overlapping circles represent contigs differentially expressed in the same direction in both comparisons.

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