Evolutionary transition from blood feeding to obligate nonbiting in a mosquito

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The spread of blood-borne pathogens by mosquitoes relies on their taking a blood meal; if there is no bite, there is no disease transmission. Although many species of mosquitoes never take a blood meal, identifying genes that distinguish blood feeding from obligate nonbiting is hampered by the fact that these different lifestyles occur in separate, genetically incompatible species. There is, however, one unique extant species with populations that share a common genetic background but blood feed in one region and are obligate nonbiters in the rest of their range: Wyeomyia smithii. Contemporary blood-feeding and obligate nonbiting populations represent end points of divergence between fully fertile southern and northern populations. This divergence has undoubtedly resulted in genetic changes that are unrelated to blood feeding, and the challenge is to winnow out the unrelated genetic factors to identify those related specifically to the evolutionary transition from blood feeding to obligate nonbiting. Herein, we determine differential gene expression resulting from directional selection on blood feeding within a polymorphic population to isolate genetic differences between blood feeding and obligate nonbiting. We show that the evolution of nonbiting has resulted in a greatly reduced metabolic investment compared with biting populations, a greater reliance on opportunistic metabolic pathways, and a greater reliance on visual rather than olfactory sensory input. W. smithii provides a unique starting point to determine if there are universal nonbiting genes in mosquitoes that could be manipulated as a means to control vector-borne disease.

Significance

The evolutionary transformation from a blood-feeding to an obligate nonbiting lifestyle is occurring uniquely within the genetic background of a single species of mosquito, Wyeomyia smithii, as a product of selection in nature. Associated genetic changes in metabolic pathways indicate a high anticipatory metabolic investment prior to consuming blood, presumably balanced by the reproductive benefits from an imminent blood meal. This evolutionary transformation provides a starting point for determining pivotal upstream genetic changes between biters and nonbiters and for identifying universal nonbiting genes or pathways in mosquitoes. If there is no bite, there is no transmission of pathogens; hence W. smithii offers a different approach to investigate control of blood-feeding vectors of human diseases.


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The authors declare no conflict of interest.

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Data deposition: Transcriptome sequence, assembly, and annotation of W. smithii is available through the National Center for Biotechnological Information (NCBI), https://www.ncbi.nlm.nih.gov/bioproject/?term=259209. The microarray data are available in the NCBI Gene Expression Omnibus repository (accession no. GSE10776). The source code for DEET can be browsed and is available for download at https://sourceforge.net/p/deet/code/ci/master/tree/.

See Commentary on page 836.

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disinterested nonbiters (FLdis) isolated from the same polymorphic Florida population. We then were able to compare DGE between avid biters and disinterested nonbiters derived from a common genetic background. We continued by comparing DGE between the avid biters (FLavid) with an obligate nonbiting population from Maine (MEonb). Selection on blood feeding, isolation of disinterested nonbiters, assays of propensity to bite, and experimental sampling of avid biters, disinterested nonbiters, and obligate nonbiters all occurred between 1,200 and 1,400 h subjective mosquito time to factor out variation due to diurnal or circadian rhythmicity. Biters were defined as females that broke the skin with their proboscis, but they were sampled before they actually consumed blood to factor out any DGE due to the presence of blood. Thus, our protocol was specifically designed to detect alterations in gene expression that occur before the intake of blood, i.e., switches associated with the anticipatory costs and metabolic adjustments of a blood-feeding lifestyle.

The objectives of this study were first to determine whether the evolutionary transformation from blood feeding to obligate nonbiting has taken place through a process of drift and correlated response to selection on other traits. We pursued this objective by testing for a positive association, or lack thereof, between DGE due to known directional selection on blood feeding in the Florida population and DGE between the avid Florida biters and the obligate nonbiting Maine mosquitoes as end points of evolution. The second objective was to determine if there are anticipatory costs identifiable from Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways that differ within and between populations representing diverse biting lifestyles. Third, we used an ongoing evolutionary process in nature as a template, the ultimate goal being to interrupt the spread of mosquito blood-borne disease by turning off the biting phenotype itself. Such a transformation is already taking place within a single species in nature; we just have to discover how. Hence, this third objective is an initial investigation into the “how” for moving forward.

**Results**

We compare patterns of DGE among selected avid biters (FLavid), isolated disinterested nonbiters (FLdis), and obligate nonbiters (MEonb) and relate these results to functional metabolic pathways.

**Patterns of DGE.** As indicated above, none of the mosquitoes used to determine DGE was allowed to consume blood. Selected avid biters (FLavid) were scored as biting after fully inserting their proboscis into the skin of the host but before they took up blood. Disinterested nonbiters (FLdis) were individuals that showed no inclination to insert their proboscis into a host after being given eight opportunities to do so over a 17-d period. During this period, any females biting or attempting to bite were immediately discarded. Obligate nonbiters (MEonb) were observed throughout the same sample time in the presence of a rat; in no case did any of those mosquitoes attempt to insert their proboscis.

After seven generations of selection, the incidence of biting in the selected line doubled from 19 to 40%. We first determined DGE between the avid biters in the selected line (FLavid) with the disinterested nonbiting isolates (FLdis) (Fig. 1A). This comparison maximized the genetic differences between avid and disinterested nonbiters in the same polymorphic population. We also determined DGE between the avid biters (FLavid) and the obligate nonbiters (MEonb) (Fig. 1B). This comparison maximized the genetic differences between avid biters as a specific response to direct selection on blood feeding and obligate nonbiting as an end point of evolution in nature. The fundamental question then is: What is the association between differentially expressed genes due to known selection on blood feeding (Fig. 1A) and the differential expression of those same individual genes between selected avid biters and obligate nonbiters from a natural nonbiting population (Fig. 1B)? Specifically, are the M-values in Fig. 1B associated with the M-values in Fig. 1A? (M-values are defined in Fig. 1.) A positive association is evidence for the evolution of obligate nonbiting being due to selection in nature; the lack of a significant association.

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Fig. 1. M/A plots of DGE of contigs and singletons from two comparisons. (A) DGE between individuals selected for avid biting (FLavid) and isolated disinterested nonbiters (FLdis) from the polymorphic FL population. (B) DGE between the same individuals from the avid biting line (FLavid) and individuals from the obligate nonbiting population (MEonb). Each M/A plot (Si Microarray Platform) represents two replicates of two dye swaps. On a two-dye microarray, one treatment is labeled with a green dye and the other with a red dye so that the difference in fluorescence between the two dyes represents the difference in gene expression between the two treatments; hence, “M” (for “minus”) = log(red) – log(green). To account for differences in red vs. green dye fluorescence, the dyes are “swapped” between treatments in a separate comparison. “A” symbolizes the average log intensities of the two dyes: 1/2 [log(red) + log(green)].

Bradshaw et al.

1010 | www.pnas.org/cgi/doi/10.1073/pnas.1717502115
or a negative association is evidence for the evolution of nonbiting being due to drift or to a correlated response to selection on other traits.

To illustrate the correlation between DGE due to selection for avid biters and due to oblate nonbiting, we developed “Quad” plots (Fig. 2) that show gene-by-gene M-values from the two M/\(A\) plots in Fig. 1. In Fig. 2, the horizontal axis shows the M-values from DGE between avid biters (\(FL_{avid}\)) vs. disinterested nonbiters (\(FL_{dis}\)) (Fig. L4); the vertical axis shows the M-values from DGE between avid biters (\(FL_{avid}\)) vs. obligate nonbiters (\(ME_{onb}\)) (Fig. 1B), but subject to the restriction that the false-discovery rate (24) must be \(q < 0.01\) for both M-values. Each gene is represented only once in the Quad plot: The \(x\) axis constitutes the M-value of a gene predicted from direct selection on blood feeding, and the \(y\) axis constitutes the M-value of the same gene observed between the end points of evolution between avid and obligate nonbiters. The DGE on the lower left-to-upper right diagonal indicates a positive association between direct selection on blood feeding and the evolution of oblate nonbiting. The DGE on the lower right-to-upper left diagonal indicates a negative or no correlation between DGE and the evolution of oblate nonbiting. Of the 21,618 genes identified by the differentially expressed sequence exploration tool (DEET), a broadly useful discriminating algorithm developed in our laboratory (SI-DEET: Refinement of the W. smithii Transcriptome), 1,459 met the criteria for inclusion in the Quad plot (SI Collection and Rearing), and 95% of the genes in the Quad plot fell along the axis of positive association.

qPCR Verification. Only two of 15 genes (\(actin\) and \(eip71cd\)) deviated from the expected microarray quadrant and the observed quadrant determined by qPCR data (Table S2). In this study, we compared pathways and genes within those pathways only when two or more genes were differentially expressed in the same direction.

Functional Metabolic Pathways. Among the differentially expressed genes in the Quad plot (Fig. 2), KEGG pathway enrichment analysis (25) identified seven metabolic pathways that differed significantly at \(P < 0.05\) following \(P\)-value adjustment using the sequential Bonferroni correction (Fig. 3).

Ribosome, spliceosome, ribosome, and proteasome proteins. DGE of eight of nine proteasome proteins, of all 19 spliceosome proteins, and of all 53 ribosome proteins was associated with blood feeding (Fig. 3 A–C). These results show that, relative to nonbiting, blood-feeding females are investing in protein degradation (proteasome), in posttranscriptional RNA editing (spliceosome), and in the translation of edited RNA into proteins (ribosome).

Phototransduction. Of the 11 differentially expressed phototransduction proteins on the microarray (Fig. 3D), nine were associated with nonbiting; the seven furthest from the origin all belonged to the actin family of genes; the other two were calcium-binding proteins involved in lipid metabolism and oxidoreductase activity. Two were associated with blood feeding: Arrestin-2 and \(\beta\)-adrenergic-receptor kinase (\(Gprk\)), whose joint action interferes with metabolic processes, thereby attenuating sensitivity to light (26). The attenuation of light sensitivity in the blood-feeders prompted a query for odorant-related proteins. Of the 21 contigs/singletons associated with odorant response in the \(W.\) smithii transcriptome, eight were differentially expressed, and all were associated with blood feeding (Table S1). These results show that there was an attenuation of light sensitivity coincident with an increase in odorant receptivity in blood-feeding females relative to nonbiting females.

Pyruvate metabolism. In the pyruvate pathway seven differentially expressed genes were associated with nonbiting. There was also one gene associated with blood feeding and one orthogonal to the bite/no-bite diagonal (Fig. 3E). The sole gene in the biting quadrant (lactoylgutathione lyase) and the off-diagonal gene (alddehyde dehydrogenase family seven member A1) were remotely located in the pyruvate pathway and were not connected with each other (Fig. S1). DGE in the pyruvate nonbiting quadrant (Fig. 4) clustered first around the conversion of pyruvate to acetyl-CoA. The generation of acetyl-CoA provides a basis for both the synthesis and degradation of fatty acids and entry into the citric acid cycle by combining with oxaloacetate (Fig. 4). However, neither enzymes linking acetyl-CoA to fatty acid metabolism nor enzymes linking acetyl-CoA to the citric acid cycle were differentially expressed. Second, pyruvate is potentially linked to hypoxia through lactate or alanine, but, again, neither of the linking enzymes was differentially expressed. Third, up-regulation of oxaloacetate from malate in nonbiters potentially signals gluconeogenesis through the action of phosphoenolpyruvate carboxykinase (PEPCK), which was up-regulated, but not from pyruvate. This observation that the gene encoding pyruvate carboxylase was not up-regulated. Up-regulation of oxaloacetate also potentially enhances oxidative phosphorylation through the citric acid cycle, but the key linking enzyme, citrate synthetase, was not up-regulated. In combination, acetyl-CoA and oxaloacetate constitute gateways into fatty acid synthesis and degradation, oxidative phosphorylation, and gluconeogenesis, but the linking enzymes to these processes also were not up-regulated. Hence, the overall picture is one of increased preparedness to reallocate energy metabolism in diverse directions in nonbiting females but not to commit directly to any one of those directions.

Purine and caffeine metabolism. KEGG pathway analysis showed overlapping purine and caffeine pathways. DGE involving purine metabolism (Fig. 3F) includes three genes overlapping with caffeine: Uric acid oxidase is associated with nonbiting, xanthine dehydrogenase is associated with blood feeding, and a paralog of xanthine dehydrogenase catalyzes the generation of methyluric acid (4 N per molecule). In sum, the overlap in DGE between purine and caffeine pathways involves the excretion of nitrogen.

DGE involving purine metabolism was divided between nonbiting (eight genes) and blood feeding (11 genes) with two genes...
in a quadrant off the blood-feeding/nonbiting diagonal (Fig. 3 and Fig. S2). Among nonbiters, differentially expressed genes involved inosine monophosphate (IMP) biosynthesis. IMP forms a branch point leading either to RNA and DNA or to ATP synthesis. Hence, DGE in nonbiting females indicates increased preparedness, relative to biting females, to allocate resources to alternate pathways, rather than directing metabolism to one in particular.

Among blood feeders, differentially expressed genes were directed toward the regulation of protein phosphorylation and proteolysis, cell cycle, nucleotide biosynthesis, DNA and RNA polymerases, and female germline ring canal formation (Table S3). These results show a preparation to break down proteins, presumably those ingested from the blood meal, and to initiate cell proliferation and ovarian development.

Protein Analysis Through Evolutionary Relationships (PANTHER) Overrepresentation Tests. To interpret the gene-expression results in terms of known and phylogenetically conserved gene functions, plus genetic regulatory and metabolic pathways, we analyzed gene lists for enrichment of biological and molecular gene functions (Table S4). Biological processes included genes involved in translation and organonitrogen processes; molecular processes included genes structurally involved in ribosomes and molecular activity; proteins included genes involved in ribosomal and RNA-binding proteins. All these enriched functions were overrepresented in blood-feeding mosquitoes compared with their nonbiting counterparts, thus reinforcing the results from the KEGG pathway analysis that indicated an increased preparedness in blood feeders to deal with the exigencies of altered nitrogen balance and to advance cell-cycle activities through enhanced translation and ribosomal structure and function.

Discussion
Directional selection on blood feeding in a low-biting polymorphic population resulted in a direct response to selection, doubling the propensity to bite within seven generations and confirming that biting is a highly heritable trait. Response to selection also resulted in DGE between selected avid biters (FLavid) and isolated nonbiters (FIdis) in the same southern population. Of the DGE between FLavid and FIdis, 95% of the genes also showed DGE in the same direction between FLavid and an obligate nonbiting population (MEonb) (Fig. 2). The close association of DGE between direct response to selection and the evolutionary transition to obligate nonbiting in nature leads us to conclude that the evolution of the northern obligate nonbiting lifestyle from blood-feeding ancestors has been the consequence of selection through evolutionary time within this single, fully interfertile species.

There are contemporary taxa of mosquitoes that never bite but persist and even thrive. The selective forces leading from a blood-feeding to a nonbiting lifestyle among different species and genera of mosquitoes are lost in evolutionary time, but blood feeding is not a free lunch. The added nutritional benefits of blood feeding are balanced by both extrinsic and intrinsic costs. There are known extrinsic costs of blood feeding that are incurred in finding and surviving on a host (27–29). Consuming a
blood meal also involves intrinsic costs. First, there is a thermal shock due to imbuing a hot blood meal at ~40 °C (30, 31). Second, the breakdown of hemoglobin specifically liberates toxic heme and iron that are excreted or bound and sequestered in the peritrophic matrix (32–37). DGE within and between populations of *W. smithii*, in which we can see a tight association between direct selection on blood feeding and the evolutionary transition to a nonbiting lifestyle in a single interfertile species, contrasts two broad patterns of gene expression: direct anticipatory costs and flexible metabolic opportunism.

First, the direct costs of consuming a blood meal in biting females of *W. smithii* are evidenced by the preparation for protein degradation by up-regulation of protein phosphorylation, the proteasome, and proteolysis (Fig. 3 A–C and Table S3), by a targeted increase in odorant receptors and decreased visual response (Fig. 3D), by investment in cell-cycle activity including nucleotide synthesis and DNA and RNA polymerases (Fig. S2 and Table S3), and by incipient ovarian maturation as indicated by up-regulating female germline ring canal formation (Table S3). In sum, these costs have the common theme of preparing to digest the expected blood meal and to initiate subsequent ovarian maturation. It is important to note that all these costs are incurred before blood is consumed.

Second, flexible metabolic opportunism in nonbiting females is evidenced by the up-regulation of visual odorant receptor (Fig. 3D) and by the up-regulation of metabolism leading to IMP (Fig. S2 and Table S3), which provides a branch point in purine metabolism that can lead, among other products, to DNA synthesis, nitrogen metabolism, or to the cytosolic second messengers cAMP and cGMP (38). Flexible metabolic opportunism is also represented by pyruvate-related enzymes that serve as gateways to diverse functional downstream processes (Fig. 4). Nonbiting is associated with the conversion of pyruvate to acetyl-CoA but not with the potential connection between acetyl-CoA and either fatty acid metabolism or entry into the citric acid cycle. At the same time, pyruvate in nonbiters is not directly linked to potential responses to hypoxia (lactate or alanine cycles) or to the direct generation of gluconeogenesis via the conversion of pyruvate carboxylase to oxaloacetate. Nonetheless, the potential for gluconeogenesis remains, because nonbiting is associated with the up-regulation of PEPCK, the crucial step in converting oxaloacetate to phosphoenolpyruvate in gluconeogenesis. In sum, nonbiting is associated with numerous “dogs that don’t bark”—the opportunistic potential for generating diverse downstream functional processes in response to appropriate environmental conditions.

**Conclusion**

The evolutionary transformation from a blood-feeding to an obligate nonbiting lifestyle in *W. smithii* is the product of selection in nature that has resulted in reduced anticipatory costs and the adoption of a more opportunistic lifestyle. The major remaining question is what specific upstream genes regulate the alteration of the associated metabolic processes that control this transformation. Mosquitoes have been called the “world’s most dangerous animal” (39) due to their ability to transmit heinous pathogens causing debilitating or lethal diseases in humans and livestock (1, 40–43). Identification of key genes responsible for the evolution of an obligate nonbiting lifestyle provides the potential to mitigate mosquito-borne diseases, because, if there is no bite, there is no disease transmission. At the genetic level, we have to determine the means by which this transformation has taken place and whether it leads to the identification of universal nonbiting genes or to universal target genetic pathways in mosquitoes. Is this determination possible? It should be: The genetic and genomic tools are available. We know that Mother Nature has done it, not only in the remote evolutionary past among different genera or species, but also today, as seen among the fully interfertile populations of a single species of mosquito.

### Materials and Methods

**Larvae of a low-biting population of *W. smithii* were collected from Florida (30%), and larvae of an obligate nonbiting population were collected from Maine (48%).** Stock populations of both the Florida (FL) and Maine (ME) populations were maintained under standard conditions (SI Collection and Rearing). From the wild-caught FL individuals, 14,000 larvae were reared to adulthood and selected for avid biting for seven generations (SI Directional Selection on Blood Feeding). The line selected for avid biting (Flavid), the low-biting FL stock population, and the obligate nonbiting ME stock population were synchronized in diapause. After 1 mo in diapause, four replicates of 1,200 larvae each of FL, and ME were reared to adulthood in 12 separate adult cages (four replicates of three treatments). Starting 5 d after first adult female emergence, each of the 12 cages was offered a female Sprague–Dawley rat (Harlan Laboratories). Rats were anesthetized with a ketamine/xylazine mixture and offered as a blood source according to University of Oregon Institutional Animal Care and Use Committee protocols 10, 11, and 13–15. Rats were offered to mosquitoes three times a week for 15 min between 1,200, and 1,400 h subjective mosquito time to minimize diurnal or circadian rhythmicity on transcriptional profiles. The order in which rats were introduced into cages was rotated among the 12 cages during each sampling episode. Each cage was observed individually and continually during the 15 min that the rat was present.

**Flavid.** From each replicate of the Flavid-selected line, any female that inserted her proboscis into the skin of the rat was scored as an avid biter (Flavid).

**Fldis.** From each replicate of the FL stock population, any female that inserted her proboscis into the rat was scored as a biter, removed from the cage, and discarded. This process continued for 17 d, allowing females eight separate opportunities to bite. At the end of 17 d, 300 of the remaining females from each cage were scored as disinterested biters (Fldis). None of the disinterested females had made any attempt to bite.

**MEonb.** In no cage did any of the females from the ME stock population attempt to bite. Samples of a few females were removed from the cage three times a week over a 17-d period and scored as obligate nonbiters (MEonb).

Once scored as an avid biter (Flavid), a disinterested biter (Fldis), or an obligate nonbiter (MEonb), females were flash frozen in 95% ethanol on dry ice and decapitated, and their heads were homogenized in cold TRI Reagent (TR-118; Zymo Research) and stored at ~80 °C. Three hundred
heads from each of the four replicates of each of the three treatments were sampled and processed as described in SI Tissue Collection and RNA Isolation.

Gene expression was measured using a custom W. smithii microarray (SI Microarray Platform) with two replicated dye swaps (SI Microarray Hybridization). Comparison among treatments used the limma package of Bioconductor (SI Microarray Analysis). Orthologs and paralogs/plpse variants were scored using the DEET pipeline developed in our laboratory (SI DEET).

Refinement of the W. smithii Transcriptome and SI Distribution of Paralogs and Splice Variants). DEG was verified with qPCR (SI qPCR Verification). Finally, differentially expressed pathways were assessed using KEGG and PANTHER analyses (SI KEGG and PANTHER).

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