The Genetic Architecture Underlying Biting in the Pitcher-Plant Mosquito, *Wyeomyia smithii*

by

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Title: The Genetic Architecture Underlying Biting in the Pitcher-Plant Mosquito, *Wyeomyia smithii*

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By taking multiple blood meals, a female mosquito is ideally suited as a vector for transmitting blood-borne diseases. With the ultimate goal of preventing pathogen transmission by mosquitoes, we determined the genetic architecture underlying blood feeding (biting) in *Wyeomyia smithii*. We crossed an obligately non-biting northern population and a biting southern population of *Wyeomyia smithii* and assayed the propensity to bite among the biting parent, F1, F2, and backcross generations. A Joint-Scaling test revealed that the evolutionary transformation from a southern, blood-feeding population to a northern, obligate non-biting population involved additive and dominance, but not maternal or epistatic effects. This result contrasts markedly with earlier findings in other phenotypes that epistasis plays a consistent role in the evolution of seasonal adaptation in this species.

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Introduction:

The ability of mosquitoes to transmit diseases is dependent on a physiological mechanism enabling females of most species to take multiple blood-meals to complete ovarian maturation, especially vitellogenesis.[1,2] Effective transmission of pathogens occurs during the biting process when female mosquitoes inject saliva into the host prior to imbibing blood.[1,3,4] The saliva contains proteins that prevent blood clotting and reduce the sensation of the bite.[3] As a result, female mosquitoes can imbibe pathogens from the blood of one host and later transmit them to new hosts during successive blood-meals.[1] Throughout the world, mosquitoes transmit a wide range of pathogens from human diseases such as malaria, yellow fever, and dengue to domesticated animal diseases such as heartworm and equine encephalitis.[1] The destruction caused by the spread of these diseases amounts to approximately 1 million human deaths every year.[5] In 2010 malaria, a pathogenic protozoan, killed approximately 660,000 people, mostly in underdeveloped parts of Africa and Asia.[6] Severe dengue, caused by a *Flaviviridae* virus, is the leading cause of death among children in parts of Asia, Africa, and South America.[7,8] The recent Zika virus outbreak, another member of *Flaviviridae*, is associated with an increase in the occurrence of fetal microcephaly (reduced brain development) likely due to the incorporation of the virus into the fetal genome.[9,10] While mosquito-borne diseases are more prevalent in less-developed countries, they continue to be major issues in global health, requiring new methods to prevent the spread of both the diseases and their vector.

Understanding the genetic basis for blood-feeding provides a novel avenue for interrupting the biting phenotype. With most mosquito systems, this research is impractical, if not impossible, as all females of a given species either require a blood-meal for repeated ovarian cycles or obligately refrain from biting entirely. Only the pitcher-plant mosquito, *Wyeomyia smithii*, naturally exhibits the necessary variation in blood-feeding between geographically distinct but completely interfertile populations to conduct this research.[11] The range of *W. smithii* extends from the Gulf Coast of Florida, USA to Canada.[11,12] Previous research from our lab determined that the ancestral range of *W. smithii* was restricted by the presence of the Laurintide Ice Sheet approximately 25,000 years ago.[13] As the ice sheet receded, populations of *W. smithii* migrated northward.[13,14] The more ancestral southern populations found at low elevations below the 37th parallel display low propensities to bite (**Figure 1**). While they all lay the first batch of eggs without biting, a portion of the southern females require a blood meal for all subsequent batches of eggs. The populations north of the 37th parallel and at high elevations in the southern Appalachians are obligately non-biting for multiple batches of eggs. Since *W. smithii* spend their pre-adult lives in the water-filled leaves of the purple pitcher-plant, *Sarracenia purpurea,* we use pitcher-plant leaves to replicate their natal habitat to stimulate egg-laying.[12,15] Using specialized climate-controlled rooms that vary heat, humidity, and light based on natural daily patterns, we can accurately replicate field conditions in the laboratory. The ability to replicate *W. smithii*'s environment under controlled conditions combined with the occurrence of both biting and obligate non-biting habits within a single species provides an ideal system for identifying the genetic architecture responsible for the biting phenotype in mosquitoes.

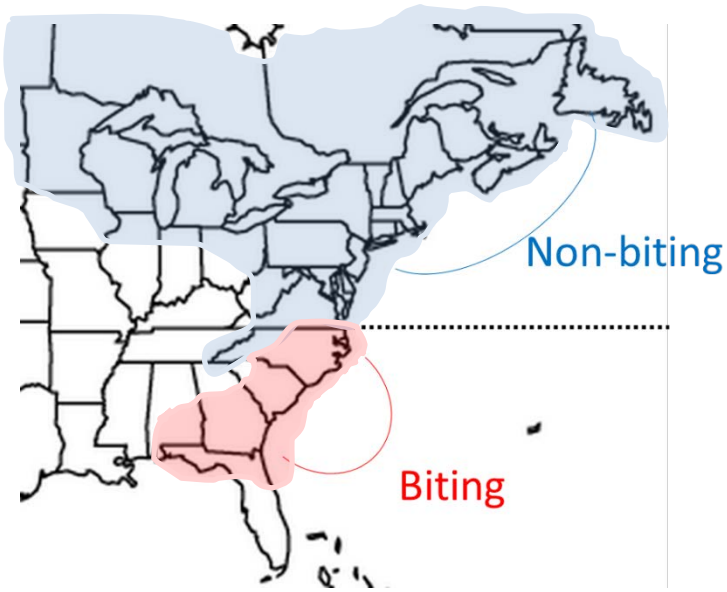

FIGURE 1: Map depicting the general range of *Wyeomyia smithii.* The region in blue indicates non-biting populations and the region in red indicates biting populations. The dashed line represents the 37th parallel.

Initially, the heritable basis of the biting phenotype was verified by selecting for vicious biting from a natural southern population with a low propensity to bite. Initially, 20% of the females bit in the original Florida population. The propensity increased to over 80% of the females after fourteen generations of selection for biting. This response to selection demonstrated that the biting mechanism is genetically based and highly heritable.

In an attempt to isolate the biting genes in a non-biting genetic background, the biting phenotype from a southern population was introgressed into a non-biting northern population. Southern biting females were released into a cage with only northern males. From their offspring, only the biting females were used to produce the next generation, again by crossing with northern males. Following this scheme, the portion of the genes from the northern population would increase and the portion from the southern

population should decrease until only the biting genes and a small number of tightly linked genes remained in an otherwise northern genetic background. Under an additivity model in which alleles within a locus act independently, and without selection, propensity to bite would be expected to decline by half each generation. However, despite selection, the biting phenotype was lost more quickly than expected, by approximately 66% each generation (**Figure 2**).

From the introgression data, we hypothesized that non-additive genetic effects, such as maternal effects, dominance, and epistasis, may play a role in the genetic architecture underlying biting. Maternal effects are phenotypic responses in the offspring that are affected by the genetic code of the mother. For example, in *Drosophila,* a hormonal cascade for proper body segmentation during development is initiated by hormones from the mother.[16] Dominance is defined as the interaction of different alleles within a locus such that in the heterozygote, one allele has a greater effect on the phenotype than the other allele. Epistasis is the interaction of alleles between loci, also commonly referred to as gene-gene interaction. Epistatic effects occur when multiple genes interact to produce a particular trait.

To test our hypothesis, we employed a quantitative genetic approach using line crosses between a northern nonbiting population and a southern biting population. The line cross design generated multiple lines of descent with varying degrees of relatedness to reveal the additive, dominance, maternal, or epistatic effects on the evolution of biting.

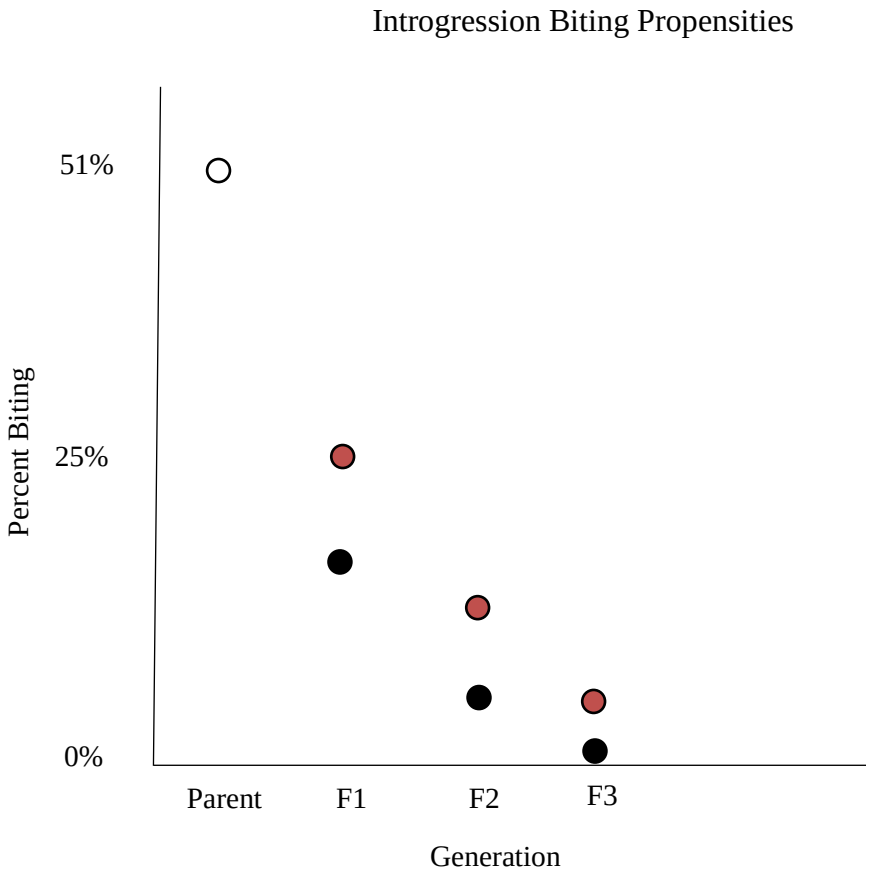

FIGURE 2: Biting propensities during three generations of introgression and selection. The open circle shows the biting propensity of the parental population. The orange circles represent the expected decline in biting based on an additive genetic effects without selection, and the black circles represent the actual propensities during three generations of introgression. The graph shows a decline of approximately 66% in biting propensity each generation even under selection. Acknowledgements: Nicholas DePatie and Jackie Houser.

Methods:

All populations were reared using a standard rearing protocol. The parental populations while in the larval stage were kept in diapause in a short-day room under a light-dark cycle of L:D = 8:16 hours at 21°C, and fed *ad lib* larval food (ground brine shrimp and gerbil food suspended in tetracycline-treated water) every week. Thirty-five larvae were kept in 150 x 25 mm petri dishes. To stimulate resumed development, the larvae were placed in a long-day room with a L:D = 18:6 hours. This amount of light is interpreted by all populations of *W. smithii* as an unambiguous long-day, triggering the resumption of development.[12] Additionally, the long-day room features a temperature and humidity cycle that mimics the pattern of a summer day. The room fluctuates the temperature following a smooth sine wave with a maximum of 35^{o}C and a minimum of 12^{o}C that lags behind the light cycle by 3 hours. Relative humidity was programmed for a constant 80% and varied from 75-100% in the room. Humidity was likely higher in

FIGURE 3: Example cage set up. Note the raisins on the top mesh surface, the *S. purpurea* leaf in the left corner and the pupae dish in the back right corner. The black dots are *W. smithii* adults.

the adult cages, since they were maintained with a water-soaked absorbent cage bottom and experienced reduced air circulation.

While exposed to long days, the larvae were fed on a weekly basis as described previously. After approximately two weeks, the larvae began to pupate. The pupae 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 fifty per dish. After four days, pupae with darkening under the dorsal region of the cuticle caused by wing development were ready to emerge as adults (eclose). These pupae were placed into the appropriate adult cage. The discarded pupal cuticle (exuviae) were collected every Monday, Wednesday, and Friday. The sex ratio of the mosquitoes in each cage was determined from the collected exuviae. The adult cage (**Figure 3**) was constructed from a 15 L food-safe bucket. The cage was fitted with two mesh sides and a mesh top to easily observe mosquitoes. Two fabric tubes were attached to the other sides to allow access into the cage while preventing mosquitoes from escaping. The bottom of the cage was removable to facilitate egg collection and lined first with food-safe paper towels and second with a sheet of Whatman 3 mm chromatography paper to facilitate collection of any eggs laid on the cage floor. Water was then added to the cage bottom to maintain humidity in the cage. A freshly opened leaf from the host plant was placed in each cage to stimulate the females to lay eggs. Approximately twenty grams of partially rehydrated raisins were placed on top of the cage as an adult carbohydrate source.

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 five days the hatched larvae were collected from the source dish and placed in groups of thirty-five into clean dishes with 1.08x10-1 mg/ml tetracycline water and dilute larval food. These dishes were labeled with the population and the date of egg collection. 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. After egg laying and hatching were complete, the larvae were randomized by

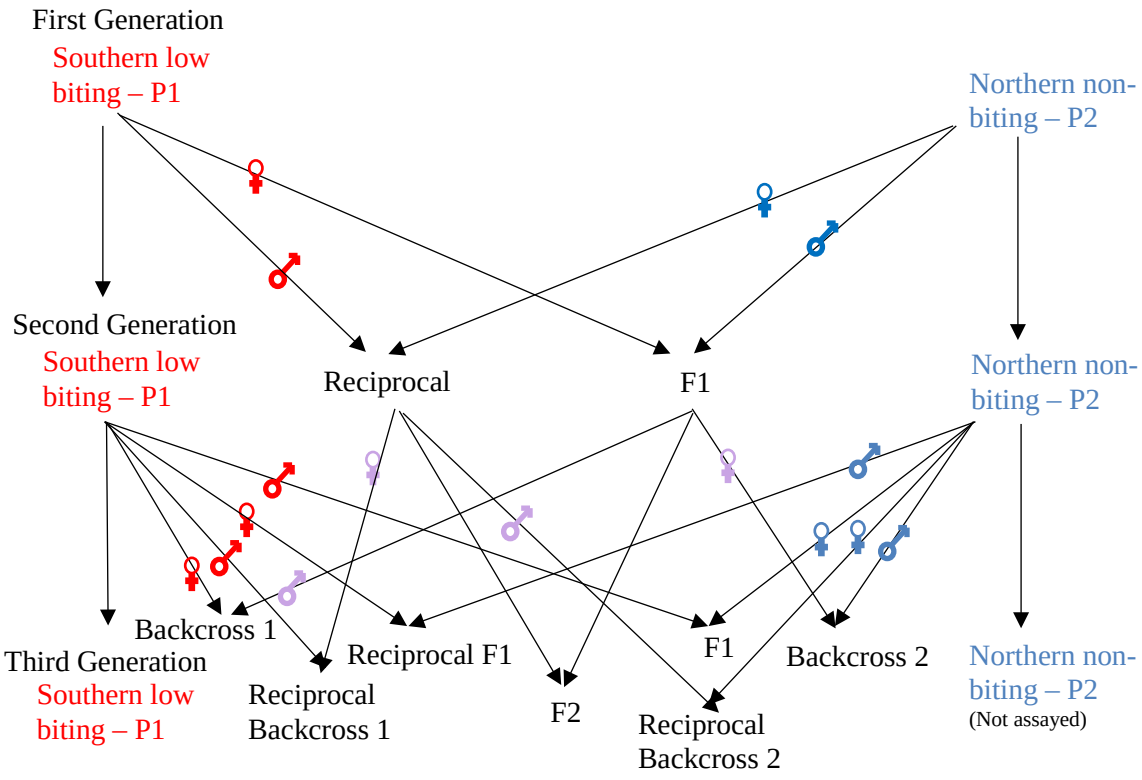

FIGURE 4: The northern x southern line cross design. Arrows labeled with ♂ indicate the contribution of male genetics from a population. Arrows labeled with ♀ indicate the contribution of female genetics from a population. Unlabeled arrows indicate both male and female genetic contribution from a population.

combining all the dishes in a large pan and then haphazardly redistributing the larvae into new dishes to prevent selection by age. After randomization, the generation was transferred to the long-day room to stimulate resumed development. The resulting adults were used to produce the next generation.

The line crosses involved two generations following the standard mosquito rearing protocol to produce the eight experimental populations (third generation) to assay for biting propensity. The specific crosses (**Figure 4**) were achieved by examining individual pupae for sexually dimorphic features to separate based on sex. 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 apparent in the abdomen, were counted and discarded. Propensity to bite was scored as:

$$p = b \div n$$

In the equation, p is the propensity to bite, b is the number of eclosed females that bit, and n is the total number of eclosed females. We then used the Joint-Scaling test to sequentially determine the role of additive, dominance, maternal and epistatic effects based on the biting propensities of the eight crosses.[14,17,18] The Joint-Scaling test evaluates the goodness of fit between the data and progressively more complicated models of genetic architecture.[18,19,20] It uses propensity 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: [21]

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

In the equation, *p* is the propensity to 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. The models we used were designed in a cumulative fashion, such that we move from an additive model to an additive-dominance model and so on. We would not discard the previous term because we would not have sufficient information to determine that it did not have a role, only that it alone was not sufficient. After evaluating a given model, the Joint-Scaling test has the power to determine significance of the separate components of that model.

Results:

Propensity to bite ranged from zero in the northern (P2) population to 0.375 in the southern population. The Joint-Scaling test rejected an additive model (χ^2 with 25.962 df = 4; $P < 0.05$) and accepted an additive-dominance model (χ^2 with 3.173 df = 3; $P = 0.366$). The departure from the additive expectation was especially apparent in the F1 and first back-cross (BC1) generations (**Figure 5**).

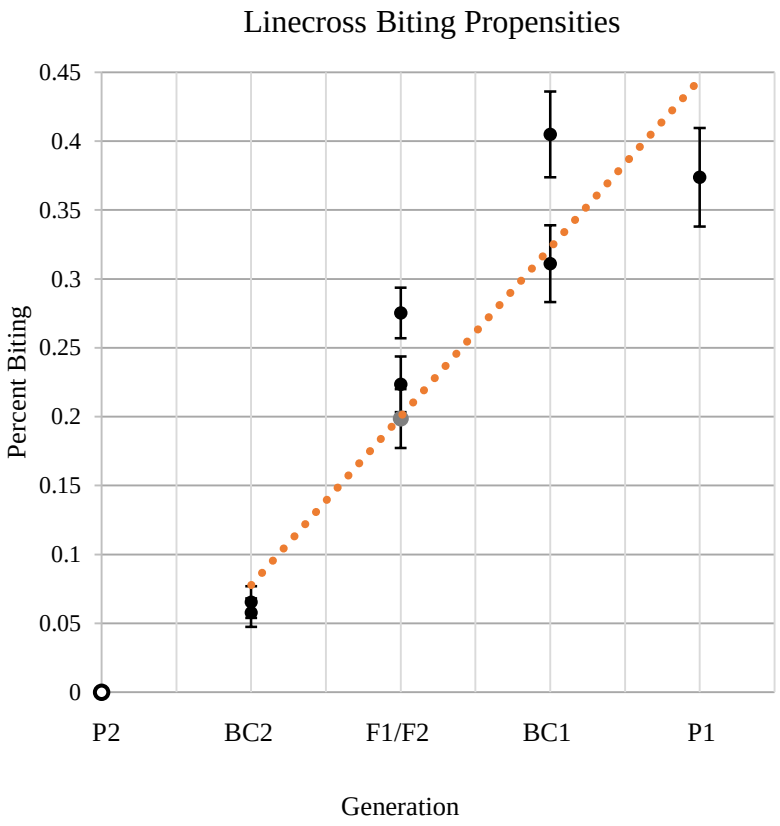

FIGURE 5: Frequency of biting in the experimental generations. The dashed orange line represents the additive expectation. The open circle denotes the northern parent population, not included in the analysis because a frequency of zero biting precludes error estimation. The black circles and grey circle (F2) denote the actual generation frequencies ± 2SE.

Conclusion:

We found that the additive-dominance model was sufficient to describe the data. In other words, the genetic difference between the northern non-biting population and the southern biting population is explained by an additive-dominance effect. Finding an additive-dominance result indicates that the genetic difference between the two populations likely involves multiple alleles at one or more loci, but provides no evidence for allelic interaction among loci (epistasis). This result stands in marked contrast to the evolution of diapause and its photoperiodic control. Despite variation over the same geographic range, differences in these seasonal traits could only be explained by invoking epistasis.[14,17,18]

The apparent absence of gene-gene interactions in the genetic architecture underlying the phenotypic variation in biting means that determining the actual genes involved in the evolutionary transition from a blood-feeding to an obligate non-blood-feeding lifestyle should be a straightforward task. Unlike research on other preventative methods, we have evidence from the life-history of naturally evolved populations that targeting these genes can create a highly-specific and effective method for preventing mosquito-borne disease transmission without changing the fitness of mosquito populations.[22]

Glossary:

Additivity: The independent expression of alleles at a single locus of a diploid organism.

Cell: The basic functional unit of life which essentially acts as a membrane bound reaction container.

Chromosome: A relatively organized unit of DNA stored in a cell.

Diapause: Suspension of development. In *W. smithii,* diapause is initiated under short day length conditions and terminated after a particular amount of long day exposure (which varies across populations based on latitude and altitude).[12,14,17]

Diploid: Organism with two copies of each chromosome.

DNA (**Deoxyribonucleic acid**): The storage molecule containing the blueprints (genes) found in all living organisms.

Dominance: Interaction between alleles at a single locus of a diploid organism in which the heterozygote phenotype is biased towards one of the homozygotes.

Dorsal: The orientation referring to the back or upper side of a given organism or specimen.

Eclose: The emergence of an arthropod from the pupal cuticle.

Epistasis: The interaction between alleles at two or more loci of a diploid organism.

Eukaryote: A group of organisms all characterized by containing a membrane bound nucleus and numerous organelles within its cell or cells.

Exuvia (plural exuviae): The exoskeleton from the pupal life stage of a mosquito.

Gametes: Specialized haploid cells (eggs and sperm) for reproduction produced by sexually-reproducing, diploid organisms.

Gene: A region of the DNA that encodes for a functional RNA or protein. A gene is also considered the basic unit of heredity.

Genetics: The study of genes, heredity, and genetic variation of life.

Genetic variation: The measure or existence of multiple alleles of genes in a population.

Genome: The entire set of DNA found in an organism.

Genotype: The allele or alleles an organism possess for a given gene.

Haploid: An organism with one copy of each chromosome.

Heredity: Passing genetically based characteristics to subsequent generations.

Introgression: The movement of a gene or trait from one gene pool to another through repeated backcrossing hybridization.

Larva (**plural larvae**): The juvenile stage of many insects including mosquitoes. The larval stage may involve several developmental stages (instars) between molts.

Linkage: Genes are said to be linked when they tend to be associated with each other in successive generations (do not segregate randomly during meiosis). Generally this phenomenon is due to the proximity of two genes on the same chromosome.

Locus (**plural loci**): A physical location on the DNA.

Meiosis: A process undergone by diploid cells to generate haploid gametes.

Nucleus: A membrane bound organelle found in eukaryotes for storing DNA in a cell.

Oocyte: An immature egg cell.

Organelle: A membrane bound compartment in a cell with a particular function, such as storage, processing, or molecular digestion.

Organism: A life form of one or more cells that maintains several key processes such as energy transfer, growth, and reproduction.

Phenotype: The expression of a genotype or genotypes under the influence of the environment.

Photoperiodism: The ability to use light cues to trigger physiological events.

Population: A group of interbreeding individuals of the same species in a given area.

Propensity: In the present context, it is the percentage of females in a population that take a blood meal.

Protein: Functional molecules of the cell composed of amino acids that are encoded in the DNA and responsible for a large portion of the structure and function of organisms.

Protozoan: A group of motile, single-celled organisms with a membrane-bound nucleus (eukaryotic organisms).

Pupa (plural pupae): The life stage generally associated with metamorphosis. In mosquitoes, pupae are the last pre-adult stage during which larval structures are broken down and adult structures are assembled.

Quantitative genetics: The genetics of generally continuous phenotypes that are due to the expression of a number of different genes.

RNA (**Ribonucleic acid**): A multifunctional molecule performing tasks such as protein synthesis, structure, and transport. RNA also acts as the transfer template for turning information in genes into proteins.

Vitellogenesis: The production and deposition of yolk (nutrients) into the oocyte.

Ventral: The orientation referring to the underside or abdominal side of a given organism or specimen.

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