Aedes aegypti: an Emerging Model for Vector Mosquito Development

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Introduction

Blood feeding mosquitoes, including the dengue and yellow fever vector *Aedes aegypti*, transmit many of the world's deadliest diseases. Such diseases have resurged in developing countries and are also emerging as clear threats for epidemic outbreaks in developed countries. Recent mosquito genome projects have stimulated increased interest in the potential for arthropod-borne disease control by genetic manipulation of vector insects. Targets of particular interest include genes that regulate development. However, although the *Ae. aegypti* genome project uncovered homologues of many known developmental regulatory genes, extremely little is known about the genetic regulation of development in *Ae. aegypti* or other vector mosquitoes. Analysis of the expression and function of individual developmental genes must now be completed. This chapter describes methodology for culturing, collection and fixation of developing tissues, analysis of gene and protein expression, and knockdown of genes during *Ae. aegypti* development. The techniques described will permit detailed analyses of the functions of developmental regulatory genes and the selective inhibition of such genes during *Ae. aegypti* development. This methodology, much of which is applicable to other mosquito species, is useful to both the comparative development and vector research communities.

Background information

Ae. aegypti (Diptera: Culicidae) is a vector mosquito of medical importance. This species has a cosmotropical distribution between 20° S and 30° N latitudes and is found throughout most tropical to subtropical world regions, where it exhibits a preference for human habitats bearing standing water. *Ae. aegypti* adults (Fig. 1), which resemble the Asian tiger mosquito *Ae. albopictus*, are a medium-sized mosquito approximately 4-7 mm in length. Adults have white scales on the dorsal surface of their thorax and a dark brown to black abdomen that may possess white scales. Tarsal segments of the hind legs have white basal bands that form what appear to be stripes (Carpenter and LaCasse, 1955).

Males and females feed on plant nectar, but females possess piercing-sucking mouthparts adapted for acquiring vertebrate blood meals prior to oviposition. Females blood feed mainly

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at dusk and dawn and primarily on human hosts. Adult females can produce up to five batches of eggs during their lifetime and lay 100-200 eggs per batch. Although eggs are typically laid on damp surfaces, eggs can survive desiccation for several months (Clements, 1999). Mosquitoes in the tribe Aedini living in temperate areas typically overwinter in a diapaused egg stage that is resistant to freezing and which lasts until spring, when they hatch as larvae. The four larval stages, as well as the pupal stage, are aquatic. Much of the larval stages, which last at least four days, is spent at the water surface, as larvae need to breathe, but larvae swim below the surface when they feed on organic particulate matter such as algae and other microscopic organisms in their aquatic habitats. Following the fourth instar, *Ae. aegypti* enter a mobile, non-feeding pupal stage which lasts approximately two days. The adult life span of this insect varies depending on environmental conditions, but ranges from two weeks to a month (Christophers, 1960; Clements, 1999; Foster and Walker, 2002).

Ae. aegypti is commonly referred to as the yellow fever or dengue fever mosquito, as it is responsible for transmission of both diseases. Prior to 1900, *Ae. aegypti* had largely escaped recognition as a world species. However, following the turn of the 20th century discovery that *Ae. aegypti* and other mosquitoes transmit human disease, numerous collections of *Ae. aegypti* from many tropical regions were established (Christophers, 1960). *Ae. aegypti* is the primary vector for the yellow fever virus, which infects 200,000 people and results in 30,000 deaths annually. Yellow fever occurs primarily in tropical regions of Africa, as well as in parts of South America. The severity of this illness ranges from a self-limited febrile illness to severe hepatitis with hemorrhagic fever. *Ae aegypti* is also the primary mosquito vector for the transmission of dengue viral infections, which can result in dengue fever, a nonspecific febrile illness which is the most widespread and significant arboviral disease in the world. Dengue virus is also the etiological agent of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which are severe and sometimes fatal forms of the disease. Dengue is a resurgent disease worldwide in the tropics, and DHF has been reported from 18 countries in the Americas. Dengue virus is presently a threat to over 2.5 billion people, with an annual incidence of approximately 50 million cases and 500,000 cases of DHF/DSS resulting in ~24,000 deaths annually (http://www.cdc.gov).

A number of factors contribute to the present inability to prevent or control mosquito-borne diseases. Although an effective vaccine for yellow fever exists, vaccination distribution is problematic, and outbreaks often occur in regions where vaccinations are not available. A vaccine for dengue does not exist, and dengue epidemics are becoming larger and more frequent. In addition to poor progress in vaccine development and distribution, the emergence of insecticide resistance in mosquito populations, a general lack of support for mosquito control programs, and increased global travel contribute to the prevalence of diseases transmitted by mosquito vectors (http://www.cdc.gov). It is therefore increasingly critical to study the biology of vector mosquitoes such as *Ae. aegypti*. In particular, the developmental biology of vector mosquitoes, the focus of this chapter, has been grossly understudied.

The *Ae. aegypti* genome project (Nene *et al.*, 2007) revealed homologues of many known developmental regulatory genes. Characterization of the function of these genes could reveal novel strategies for vector control. However, studying the development of this species has been a challenge. Christophers (1960), indicated that "the eggs of *Ae. aegypti* are not the most suitable form on which to study mosquito embryology." In fact, in his book, which remains the most comprehensive description of *Ae. aegypti* biology to date, he offered a description of *Culex molestus*, rather than *Ae. aegypti* development. The chorion and serosal cuticle of *Ae. aegypti*, which serve as barriers to fixatives, probes, and antibodies, has made working with this species a challenge in the past. However, we offer here a series of protocols that make analysis of *Ae. aegypti* development manageable. Given the many known advantages of this system (Severson *et al.*, 2004a), these protocols, in combination with recent advances in the

study of genetics and genomics in this species (Chen *et al.*, 2008), are helping to establish *Ae. aegypti* as an emerging model organism for vector mosquito development.

Sources and Husbandry

Ae. aegypti is relatively simple to grow in culture (Protocol 1, Fig. 2) and has therefore been the subject of a wide range of laboratory investigations. Other mosquito species are often difficult to adapt to laboratory culture and require constant maintenance following successful introduction to the lab. This is because the life cycles of most mosquito species are continuous, with eggs typically being laid on the water surface and hatching soon thereafter. In contrast, *Ae. aegypti* preferentially oviposit away from the water surface and are tolerant to desiccation, an adaptation which allows for collection of eggs on artificial substrates such as paper towels and subsequent storage for several months. Stored eggs can later be induced to hatch in deoxygenated water. Furthermore, mosquitoes collected in the field can readily adapt to laboratory rearing conditions and can even successfully reproduce following single pair matings (Severson *et al.*, 2004a). Given the advantages of this system, many strains have been reared in laboratory settings. Although the University of Notre Dame initially served as the World Health Organization's repository for *Ae. aegypti* strains, the ever growing number of strains are no longer maintained in a central location, but can be acquired from the individual laboratories in which they are studied.

Related Species

Sequencing of the *Ae. aegypti* (Nene *et al.*, 2007) and *Anopheles gambiae* (Holt *et al.*, 2002) genomes was an important advancement, as these species represent significant members of two medically important mosquito subfamilies, Culicinae and Anophelinae. Arbovirus and lymphatic filariasis transmission is largely associated with Culicinae, while Anopheline mosquitoes are the primary vectors for malaria transmission. The *Culex quinquefasciatus* (dominant lymphatic filariasis vector) genome is also now available at Vectorbase (http://cquinquefasciatus.vectorbase.org). Completion of these three mosquito genomes is allowing for global characterization of sequence conservation and structure through comparative and functional analyses (reviewed by Chen *et al.*, 2008). While many commonalities exist, significant differences in the biological characteristics and genomic structures of these mosquitoes have been observed (Knudson *et al.*, 2002; Rai and Black, 1999; Severson *et al.*, 2004b).

In terms of studying the developmental biology of mosquitoes, *Ae. vexans* is probably the most carefully described Aedine mosquito (Moretti and Larsen, 1973). Although these descriptions will be of use to those studying *Ae. aegypti* development, the genome of *Ae. vexans* has not yet been sequenced. As we embark upon genetic characterization of *Ae. aegypti* development, studies in *Drosophila melanogaster*, a well-established model for insect development, and *Tribolium castaneum*, an emerging model (Brown *et al.*, 2009), will be useful, as the genomes of these species have been sequenced. Mosquito homologues of genes that regulate fruit fly development are being characterized (see below). The process of generating transgenic mosquitoes has been adapted from approaches developed in *Dr. melanogaster* (see below). Techniques for fixation and preparation of developing tissues (Protocol 2, Fig. 3) developmental analyses of gene (Protocol 3, Fig. 4) and protein expression (Protocol 4, Fig. 5) in *Ae. aegypti* described here were adapted from *Drosophila* protocols (Patel, 1994, 1996). The technique for RNA inhibition (RNAi) in *Ae. aegypti* included here (Protocol 5) draws from successful RNAi studies performed in *Tr. castaneum* (Brown *et al.*, 2009).

Uses of the *Ae. aegypti* Model System

A model for mosquito-pathogen interactions

Given the relative ease of rearing *Ae. aegypti* in culture (Protocol 1), this species has been the subject of a wide range of laboratory investigations ranging from analysis of its morphology, physiology, genetics, vector competence, and molecular evolution (Clements, 1992; Severson *et al.*, 2004a, Chen *et al.*, 2008). In particular, analysis of *Ae. aegypti* has provided broad insight into the study of mosquito/pathogen interactions. Following a blood meal from an infected host, the pathogen must survive internal defense mechanisms which aim to recognize and destroy foreign entities. Variability in vector competence is attributable to genetic differences, and it is therefore critical to study vector competence in genetically well characterized mosquito species such as *Ae. aegypti* (see below). Use of *Ae. aegypti* has permitted genetic analysis of the basis of dengue vector competence. Furthermore, although *Ae. aegyti* is not a vector for human malarial parasites, it is a vector for the avian malarial parasite *Plasmodium gallinaceum*, and is thus serving as a model for the genetic basis of vector competence for malaria parasites. Likewise, although *Ae. aegypti* is not a natural vector for lymphatic filariasis, the Liverpool strain of *Ae. aegypti* is susceptible to *Brugia malayi* and *Wucheria bancrofti*, two filarial nematode strains which infect humans. *Ae. aegypti* can therefore also be used as a model for the genetic basis of vector competence to filarial worms (reviewed by Severson *et al.*, 2004a and Chen *et al.*, 2008).

Overview of *Ae. aegypti* Development

Recent mosquito genome projects (Holt *et al.*, 2002; Nene *et al.*, 2007) have stimulated increased interest in the potential for arthropod-borne disease control by genetic manipulation of vector insects. Targets of particular interest include genes that regulate development. Although the *Ae. aegypti* genome project uncovered homologues of many known developmental regulatory genes, extremely little is known about the genetic regulation of development in *Ae. aegypti* or other vector mosquitoes. Raminani and Cupp published descriptions of *Ae. aegypti* early embryology (1975) and organogenesis (1978). Their studies, which provide a firm basis for future studies which will examine developmental gene function in *Ae. aegypti*, are summarized below and in Table 1.

Embryonic development of *Ae. aegypti* (analyzed at 25° C) proceeds as follows: Fusion of the female and male pronuclei occurs approximately one hour following oviposition. The first and second mitotic divisions occur before the end of the second hour of development. Asynchronous nuclear divisions follow and result in the formation of a syncytial blastoderm by 6 hrs. of development. The germ band forms by 9 hrs., and cellularization of the blastoderm is complete by 10 hrs. of development. Columnar cells of the ventral blastoderm form the embryo proper. The amnion arises from ventrolateral cells, and dorsal blastodermal cells form the serosa. Pole cells, which begin to form at 3 hrs., remain outside the blastoderm until 11 hrs., at which time they migrate with the germ band as dorsal elongation occurs. Gastrulation initiates at 12 hrs. of development. Segments are visible at 15 hrs. and are well defined by 20 hrs. (Raminani and Cupp, 1975). Germ band shortening initiates at 30 hrs. and is complete by 40 hrs. A vitelline membrane forms by 30 hrs. of development, and lysis of the amnion and serosa is complete by 55 hrs. Dorsal closure is complete by 65 hrs. (Raminani and Cupp, 1975).

Raminani and Cupp (1978) also documented major events in organogenesis. Ventral nerve cord formation initiates at 15 hrs. of development. Labral development initiates at 18 hrs., and the labrum is visible as a distinct lobe by 35 hrs. Posterior midgut-proctodeal and anterior midgut-stomodeal invaginations form at 19 hrs. Antennal rudiments, the mandible, and maxillae are visible by 25 hrs, and the labium can be observed at 30 hrs. Foregut differentiation,

midgut, brain, and gonad formation occurs at 35 hrs. At 40 hrs., tracheal system differentiation initiates. Differentiation of the hindgut commences at 45 hrs. Head and mouthpart muscles begin to form at 50 hrs., while trunk musculature differentiates at 60 hrs. The heart is formed by 65 hrs. Formation of the hatching spine begins at 75 hrs., and embryogenesis is complete by 96 hrs.

Ae. aegypti and *Dr. melanogaster* development are compared in Table 2. The relative timing of major developmental events in both species is noted. While the relative order of developmental events is well conserved, embryogenesis proceeds more rapidly in *Dr. melanogaster*. This comparison will be useful for those who are interested in studying a particular aspect of mosquito development, or who wish to analyze the function of *Ae. aegypti* homologues of fly developmental genes.

A model for vector mosquito developmental genetics

Although we have made great advances in understanding developmental genetics in *Drosophila*, comparatively little is known about the genetic basis for development in mosquitoes and other arthropods. Studying developmental genetics in mosquitoes will surely benefit the evolutionary development research community, as data collection from non-model arthropods has been an important aspect of the recent "evo-devo" revolution. Additionally, detailed characterization of mosquito development and genes that regulate this process could precipitate new strategies for vector control.

To date, only a handful of *Ae. aegypti* developmental genes have been studied. Several groups have used a candidate gene approach to identify mosquito homologues of *Drosophila* gene products that localize to the future germ cells of the fly embryo. Expression of the *nanos* (Calvo *et al.*, 2005; Adelman *et al.*, 2007) and *oskar* (Goltsev *et al.*, 2004, Juhn and James, 2006) genes has been analyzed in several mosquito species, including *Ae. aegypti*. The expression profiles of these genes are generally well conserved, however some differences in their temporal and spatial distributions have been noted (reviewed by Chen *et al.*, 2008). These studies were completed, at least in part, through efforts to develop a drive system that will quickly spread and fix antipathogen effector genes in natural populations (Juhn and James, 2006). To this end, Adelman *et al.* (2007) showed that *nanos* control sequences demonstrated promise as part of a transposable element-based gene drive system. Thus, an evo-devo approach was applied in an effort to develop translational strategies for vector control.

In recent years, we have begun analysis of nervous system development in *Ae. aegypti* (Simanton *et al.*, 2009; Clemons *et al.*, in preparation). Analysis of mosquito nervous system development will lead to a better understanding of the developmental basis of motor function, sensory processing, and behavior, key aspects of mosquito host location. Our analyses suggest that the process of embryonic nerve cord formation, as well as the function of the axon guidance molecules Netrin (Simanton *et al.*, 2009) and its receptor Frazzled/DCC (Clemons *et al.*, in preparation) are conserved between *Drosophila* and *Ae. aegypti*. We are beginning to extend our analyses of axon guidance gene function to the developing olfactory system. The olfactory system is of particular interest to the vector community, as location of human hosts is an olfactory-driven behavior.

While detailed and thorough analysis of all aspects of mosquito development is critical, analysis of tissues that are vital to host location and the spread of infection is of particular importance. Mosquitoes rely on their olfactory systems for location of human hosts. During blood feeding, disease causing viruses and parasites are ingested and encounter the mosquito midgut epithelium, where they replicate. The infection spreads to secondary sites in the mosquito body prior to infecting the salivary glands. Following salivary gland infection, female mosquitoes are competent for disease transmission for the duration of their lives (reviewed by Catteruccia,

2007). Although analysis of mosquito olfactory, gut, and salivary gland development could have important applications for disease control, little is known about the development of these tissues in mosquitoes. We are in the process of identifying molecular markers for these tissues and are beginning to study the genetic regulation of their development.

Genetics, Genomics, and Associated Resources

Ae. aegypti is genetically one of the best characterized insect species. The development of genetic markers in *Ae. aegypti* has facilitated the tracking of groups of genes or genome segments in relation to a phenotype of interest. Linkage maps were initially generated from isozyme and mutant marker loci (Munsterman and Craig, 1979). In recent years, genetic studies in *Ae. aegypti* have benefitted from the construction of DNA-based marker technology which has resulted in the construction of genetic maps. Restriction fragment length polymorphisms (RFLPs) were used in the development of a genetic map consisting of 53 loci covering 134 cM (Severson *et al.*, 1993). This map was expanded through the inclusion of single nucleotide polymorphisms (SNPs), single strand conformational polymorphisms (SSCP), and microsatellite (SSR) markers, to cover 154 loci over 205 cM (Severson *et al.*, 2002, Chambers *et al.*, 2007). A genetic map consisting of 96 loci and spanning 168 cM was also prepared through use of SSCP analysis of random amplified polymorphic DNA (RAPD) markers (Antolin *et al.*, 1996). SSCP technology was applied toward the development of additional genetic markers from cDNA sequences (Bosio *et al.*, 2000; Fulton *et al.*, 2001). More recently, Lovin *et al.* (2009) showed that the *Ae. aegypti* genome is populated with single copy polymorphic microsatellite loci which are suitable for genetic and population studies (Hemme *et al.*, 2010).

David Severson coordinated a whole genome shotgun sequencing effort to determine the complete annotated genome sequence of *Ae. aegypti*. Preliminary annotations were developed by two independent groups (The Institute for Genomic Research and VectorBase), and a final annotated sequence was reported (Nene *et al.*, 2007). All information for the *Ae. aegypti* genome project is accessible at Vectorbase (http://aaegypti.vectorbase.org/index.php). AaegL1.2, an update to the initial gene set (AaegL1.1) was released by Vectorbase in 2009. The genome sequence assembly consists of 4,758 scaffolds spanning 1,310 million basepairs (Mbp). 15,988 genes are estimated; rules and conventions for naming genetic features in members of the family Culicidae are described (VectorBase). In recent years, VectorBase has initiated a community annotation system, a microarray and gene expression repository, and has continued to develop software infrastructure and tools for interrogating stored sequence data of *Ae. aegypti* and other arthropod vectors (Lawson *et al.*, 2009).

Technical Approaches

Generation of transgenics

The recent completion of the *Ae. aegypti* genome project (Nene *et al.*, 2007) is facilitating studies aiming to expand the current understanding of mosquito biology. Such investigations may ultimately identify new targets of vector control, lead to development of strategies for introduction of refractory traits in natural populations, or help to control the size of mosquito populations (Catteruccia, 2007). The potential for arthropod-borne disease control by genetic manipulation of vector insects has been recognized and advocated for four decades (Knipling *et al.*, 1968). The identification of genes involved in host-seeking, blood meal acquisition and digestion, reproduction, susceptibility/refractoriness to pathogens, and insecticide resistance is expected to yield novel approaches for the control of mosquito-borne illnesses (Chen *et al.*, 2008). Several genetic control strategies for mosquitoes have been proposed, including: sterile male release programs, cytoplasmic incompatibility, hybrid sterility, and population replacement. However, while several of these strategies are promising, they have not yet been

implemented successfully. Recently, considerable efforts are being directed toward applying transgenesis technology to arthropod-borne disease control, with an emphasis on population replacement (Adelman *et al.*, 2002; Moreira *et al.*, 2002; Robinson *et al.*, 2004, Chen *et al.*, 2008). A major focus of this research is to use these techniques to generate genetically-modified mosquitoes carrying transgenes that disrupt their ability to interact with hosts and transmit pathogens. These transgenic mosquitoes would be introduced into natural mosquito populations. Successful implementation of such strategies will require intensive prerelease and post-release investigations of mosquito ecology and population biology (Scott *et al.*, 2002).

Successful integration of transgenes into the germline of *Ae. aegypti* (reviewed by Adelman *et al.*, 2002; Lobo *et al.*, 2006, Chen *et al.*, 2008) has been accomplished with class II transposable elements, including *Hermes, mariner*, and *piggybac*. A high-efficiency germ-line transformation protocol has been described (Lobo *et al.*, 2006), and a helpful video protocol documenting the microinjection process is available (Jasinskiene *et al.*, 2007). Transgenes are introduced through microinjecting mosquito embryos at syncitial blastoderm stages. Fluorescent protein markers such as GFP are typically used to monitor transgene insertion. Such methodology has generated transgenic mosquitoes that may prove useful for the control of mosquito-borne illnesses, including a recently described female-specific flightless phenotype strategy for mosquito control (Fu *et al.*, 2010).

Functional analysis of genes

RNA interference (RNAi) is proving to be a useful method for functional analysis of genes in *Ae. aegypti* (Brown and Catteruccia, 2006). For analysis in adults, dsRNA is typically injected into female abdomens; a video protocol illustrating this methodology is available (Luna *et al.*, 2007). Blitzer *et al.* (2005) used RNAi knockdown strategies for functional analysis in larvae. A protocol for siRNA-mediated knockdown of genes in embryos is included here (Protocol 5). These methodologies are yielding information about genes that are important for combating mosquito-borne illnesses. For example, silencing of the *Ae. aegypti cactus* genes was found to promote developmental arrest and death of the avian malaria parasite, *Plasmodium gallinaceum* (Zou *et al.*, 2008). Xi *et al.* (2008) used RNAi strategies to functionally assess the role of the *Ae. aegypti* Toll pathway in regulating resistance to dengue virus.

Analysis of Developmental Genetics in *Ae. aegypti*

In recent years, evolutionary developmental biologists have applied knowledge of developmental genetics in *Drosophila melanogaster*, a genetically tractable model organism, to better understand development of other arthropods. These efforts typically involve the tedious process of cloning and assigning homology to genes from non-model organisms in which functional analyses are not always possible. Sequencing of the *Tribolium castenium* genome and the development of strategies for examining gene function have greatly enhanced analysis of developmental genetics in the red flour beetle (Brown *et al.*, 2009). It is anticipated that the *Ae. aegypti* genome sequence, in combination with the development of techniques for analyzing gene expression and function during development, will allow *Ae. aegypti* to emerge as an excellent model for vector mosquito development. This chapter describes methodology for culturing and egg collection (Protocol 1), fixation and dissection (Protocol 2, Fig. 3), analysis of gene (Protocol 3, Fig. 4) and protein (Protocol 4, Fig. 5) expression, and knockdown of genes (Protocol 5) during *Ae. aegypti* development. The techniques described will permit detailed analysis of the function of developmental regulatory genes and the selective inhibition of such genes in *Ae. aegypti*. These studies and methodology, much of which are applicable to other mosquito species, are useful to both the comparative development and vector research communities.

Acknowledgments

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Useful web resources

http://aaegypti.vectorbase.org/index.php Vectorbase website, which includes sequence data, expression data, images, and other additional resources pertaining to *Ae. aegypti*.

http://www.cdc.gov/Dengue/ CDC website on dengue, which includes travel/outbreak notices, prevention, symptoms, epidemiology and statistics, entomology and ecology, clinical/laboratory guidance, education and training.

http://www.cdc.gov/ncidod/dvbid/yellowfever/ CDC website for yellow fever, which includes information about prevention, vaccination, transmission, symptoms, diagnostic testing, health care provider links and references, and travel.
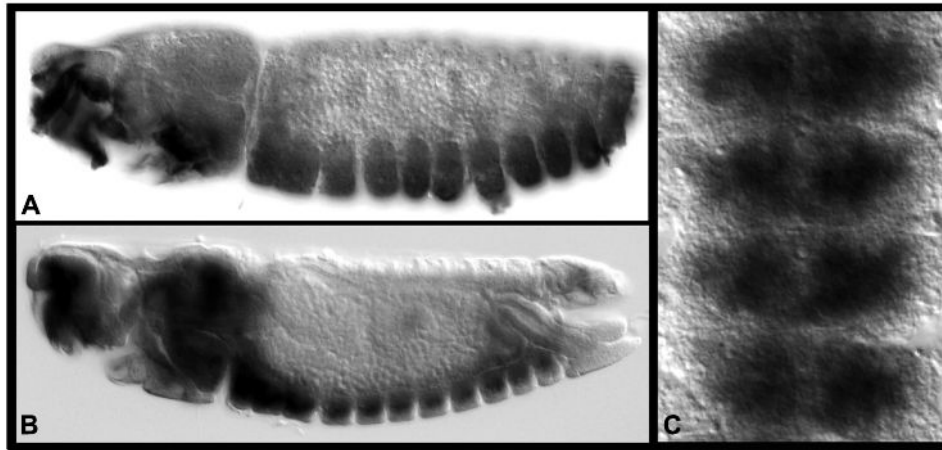
Figure 1.
Adult *Ae. aegypti* female. *Ae. aegypti* adults have white scales on the dorsal surface of their thorax as well as a dark brown/black abdomen that may also possess white scales. The tarsal segments of the hind legs have white basal bands. Photo Credit: James Gathany, CDC.
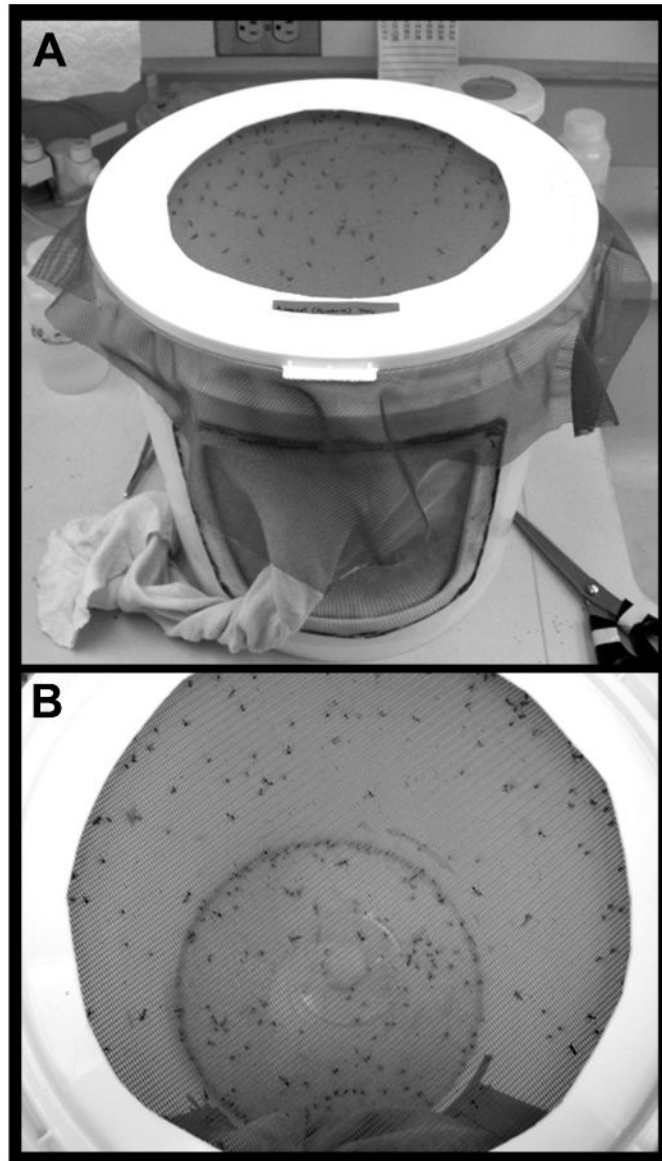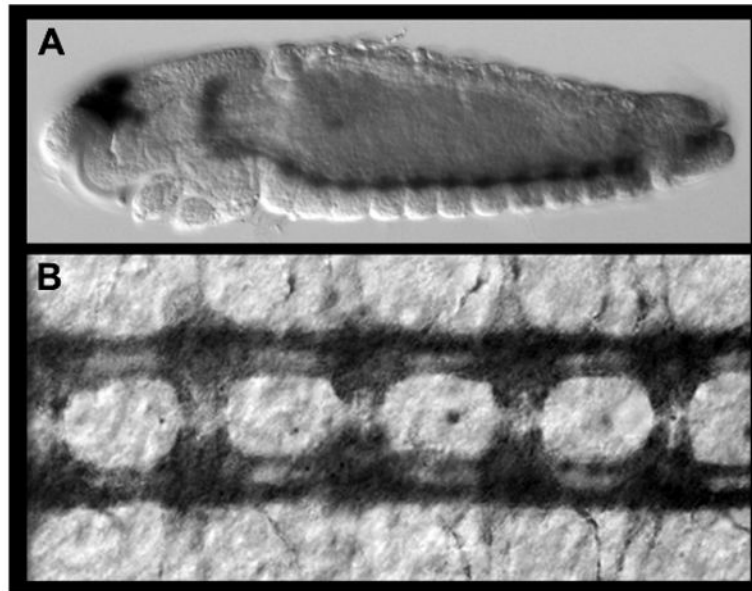
Figure 2.
Mosquito rearing cage. Methodology for culturing mosquitoes is described in Protocol 1. The cotton sleeve on the cage (seen in A) permits human access and manipulations within the cage. The netted top of the cage (top down view shown in B) allows for blood feeding prior to egg collections.
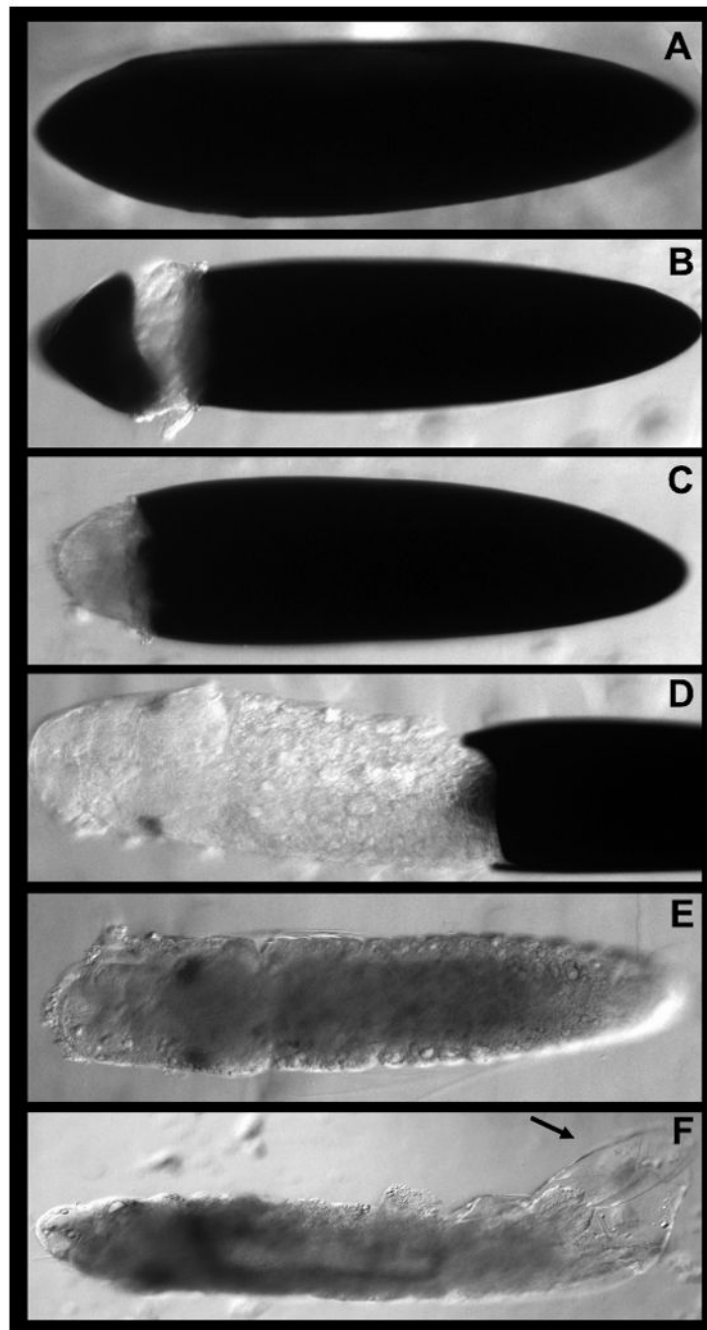
Figure 3.
Dissection methodology for *Ae. aegypti* embryos. Methods for fixing and dissecting mosquito embryos are included in Protocol 2. The process of dissecting a fixed embryo is shown in A-F (see text for details). It is critical to remove the black endochorion (A-D) and transparent serosal cuticle (arrowhead in F) in order to successfully perform gene and protein expression assays.

Figure 4.
Expression of axon guidance genes in *Ae. aegypti* embryos. (A) *fra* and *casein kinase* (B, C) are expressed ventrally in the developing nerve cord at 55 hrs. of development. Lateral views of whole-mount embryos stained with the accompanying *in situ* methodology (Protocol 3) are shown in A and B (anterior is oriented left). A filleted nerve cord is shown in C (anterior is oriented up).

Figure 5.
Protein expression analysis in *Ae. aegypti* during embryonic development. (A,B) Acetylated tubulin expression labels the axons of the developing nerve cord. A lateral view of a 55 hr whole-mount embryo stained with the accompanying immunohistochemistry methodology (Protocol 4) is shown in A (anterior is oriented left). A filleted nerve cord from a 55 hr embryo is shown in B (anterior is oriented up).

TABLE 1

Developmental Events in *Aedes aegypti* Embryogenesis (after Raminani and Cupp, 1978). The timing of major embryonic developmental events is indicated in hrs after egg laying (AEL) at 25° C.

Age (hrs AEL)	Event
0.5	Polar body formation
1	Fertilization
1-2	1st and 2nd mitotic divisions
3	Formation of pole cells and primary yolk cells
4	Peripheral migration of cleavage nuclei
5	Pole cells outside the blastoderm
6	Syncitial blastoderm
8	Cellularization of blastoderm
9	Germ band formation; secondary yolk cells
11	Pole cell entry into the blastoderm
12	Initiation of gastrulation; Migration of presumptive mesodermal cells, elongation of the germ band
14	Amnion and serosa formation
15	Segmentation of the embryo begins; Ventral nerve cord formation begins.
18	Labral lobe formation
19	Anterior midgut-stomodaeal and posterior midgut-proctodaeal invaginations form.
25	Antennae, mandibles and maxillae begin to form; anterior migration of the mouthparts begins.
30	Vitelline membrane present; germ band retraction begins; labial lobes appear.
35	Blastokinesis and dorsal closure begin; foregut differentiation and formation of the midgut begins; formation of the brain and gonads initiates.
40	Differentiation of the suboesophageal ganglion and tracheal system starts.
45	Hindgut differentiation begins; formation of the retrocerebral complex and stomodaeal nervous system initiates.
50	Breakdown of the amnion and serosa; oenocytes and fat cells are formed; muscles of the head and mouthparts begins to form.
60	Differentiation of trunk musculature
65	Heart formation
75	Formation of the hatching spine begins.
96	Embryogenesis completed

TABLE 2

Temporal Comparison of Major Developmental Events in *Dr. melanogaster* and *Ae. aegypti* Embryogenesis. The relative timing of major developmental events, measured in hrs after egg laying (AEL) at 25° C, is noted for both species. The relevant *Drosophila* stage is also indicated. While major developmental events and their relative timing is generally well conserved, embryogenesis proceeds more rapidly in *Dr. melanogaster*.

Event	*Dr. melanogaster* Age (hours AEL; stage)	*Ae. aegypti* Age (hours AEL)
Cleavage	0-1.2; 1-4	4
Pole cells form	1.2; 3	3
Syncitial blastoderm	1.5-2.2; 4	6
Cellularization of blastoderm	2.5; 5	8
Onset of gastrulation	3; 6	12
Midgut formation begins	3.1; 7	35
Germ band extension	3.3; 8	12
Nerve cord formation begins	3.75; 9	15
Foregut differentiation	4.25; 10	35
Tracheal system differentiation	5.25; late 10	40
Brain formation initiates	5.3; 11	35
Germ band retraction	7.25; 12	30
Segment formation evident	7.25; 12	15
Dorsal closure begins	9.25; 13	35
Gonad formation begins	11.4; 15	35
Completion of dorsal closure	13; 15	65

After Martinez-Arias, 1993; Raminani and Cupp, 1978