siRNA-Mediated Gene Targeting in *Aedes aegypti* Embryos Reveals That *Frazzled* Regulates Vector Mosquito CNS Development

Anthony Clemons¹,², Morgan Haugen², Christy Le¹, Akio Mori¹, Michael Tomchaney¹, David W. Severson¹,², Molly Duman-Scheel¹,²*

¹Department of Biological Sciences and Eck Institute for Global Health, University of Notre Dame, Notre Dame, Indiana, United States of America, ²Department of Medical and Molecular Genetics, Indiana University School of Medicine, South Bend, Indiana, United States of America

**Abstract**

Although mosquito genome projects uncovered orthologues of many known developmental regulatory genes, extremely little is known about the development of vector mosquitoes. Here, we investigate the role of the Netrin receptor *frazzled* (*fra*) during embryonic nerve cord development of two vector mosquito species. *Fra* expression is detected in neurons just prior to and during axonogenesis in the embryonic ventral nerve cord of *Aedes aegypti* (dengue vector) and *Anopheles gambiae* (malaria vector). Analysis of *fra* function was investigated through siRNA-mediated knockdown in *Ae. aegypti* embryos. Confirmation of *fra* knockdown, which was maintained throughout embryogenesis, indicated that microinjection of siRNA is an effective method for studying gene function in *Ae. aegypti* embryos. Loss of *fra* during *Ae. aegypti* development results in thin and missing commissural axons. These defects are qualitatively similar to those observed in *Dr. melanogaster fra* null mutants. However, the *Aa. aegypti* knockdown phenotype is stronger and bears resemblance to the *Drosophila commissureless* mutant phenotype. The results of this investigation, the first targeted knockdown of a gene during vector mosquito embryogenesis, suggest that although *Fra* plays a critical role during development of the *Ae. aegypti* ventral nerve cord, mechanisms regulating embryonic commissural axon guidance have evolved in distantly related insects.

**Introduction**

Completion of the *Aedes aegypti* and *Anopheles gambiae* genome projects uncovered orthologues of many known developmental regulatory genes in these two important mosquito vectors of dengue and malaria, respectively [1,2]. Although characterization of the function of these genes could provide insight into the evolution of insect development or potentially reveal novel strategies for vector control, extremely little is known about the genetic regulation of mosquito development [3,4]. Excellent descriptive analyses of *Ae. aegypti* embryogenesis were completed in the 1970’s [5,6], and additional developmental analyses in this species were recently published [7,8]. Still, expression of only a handful of mosquito embryonic genes has been described in *Ae. aegypti* or other vector mosquitoes [9,10,11,12,13,14,15,16]. This is likely a result of the technical challenges historically encountered by those performing developmental analyses in mosquitoes. In fact, Christophers [17], author of the most comprehensive text on the biology of *Ae. aegypti*, indicated that the eggs of this species are not the most suitable form on which to study mosquito embryology.

Given the many known advantages of studying the biology of *Ae. aegypti* [3,18], we recently published a series of protocols for the study of its development [19,20,21,22,23]. These methodologies, in addition to those published previously [9,11], will promote analysis of mosquito developmental genetics. We are presently employing these techniques to examine mosquito nervous system development. Analysis of mosquito neural development will lead to a better understanding of the developmental basis of motor function, sensory processing, and behavior, key aspects of mosquito host location.

During *Drosophila melanogaster* nervous system development, midline cells secrete guidance molecules such as Netrin (Net) proteins that regulate the growth of commissural axons [24,25,26]. The *Dr. melanogaster* Net proteins are expressed at the midline and are required for proper commissural axon guidance in the embryonic ventral nerve cord. Frazzled (*Fra*), the *Drosophila* homolog of the vertebrate Deleted in Colorectal Cancer (DCC) Net receptor, guides axons in response to Net signaling [27] and also controls Net distribution in flies [28]. Previous studies indicated that deletion of *net* and *B* or *fra* results in defective guidance of commissural axons in *Drosophila* [27,29,30]. More recent data suggest that *Drosophila* Net function as short-range guidance cues that promote midline crossing [31].

Although data support the homology of axon-guiding midline cells [16,32,33,34,35,36], homology of midline cells, which form
differently in various arthropod species (discussed in [32]) has been debated. To address whether common molecular mechanisms regulate nerve cord formation during arthropod nervous system development, we recently analyzed patterns of axon tract formation and the putative homology of midline cells in distantly related arthropods. These comparative analyses were aided by a cross-reactive antibody generated against the Netrin (Net) protein, a midline cell marker and regulator of axonogenesis [16]. Despite divergent mechanisms of midline cell formation and nerve cord development in arthropods, detection of conserved Net accumulation patterns suggests that Net-Fra signaling plays a conserved role in the regulation of ventral nerve cord development of Tetrakaonata [16]. Here, we continue to examine this hypothesis through examination of the expression of the Net receptor frazzled in both Ae. aegypti and An. gambiae. Moreover, for the first time, we use siRNA-mediated knockdown to functionally test this hypothesis in Ae. aegypti.

Results and Discussion

Development of the mosquito embryonic ventral nerve cord

A scaffold of axon pathways develop in Dr. melanogaster and give rise to the embryonic ventral nerve cord, which has a ladder-like appearance (Fig. 1D). Within each segment of the developing fruit fly embryo, a pair of bilaterally symmetrical longitudinal axon tracts are pioneered separately on either side of the midline in each segment. A number of early growth cones project only on their own side, but most CNS interneurons will project their axons across the midline in either the anterior or posterior commissural axon tracts before extending rostrally or caudally in the developing longitudinals ([24,25]; Fig 1D). Nerve cord development was assessed during mosquito embryogenesis with an anti-acetylated tubulin antibody (Fig. 1A–C). Acetylated tubulin is first detected in developing axons of the commissural and longitudinal axon pathways, including the earliest commissural axons [27]. Expression of Ae. aegypti fra (Aae fra) and An. gambiae fra (Aga fra) were therefore analyzed through whole-mount in situ hybridization at the onset of nerve cord development in both species. Ae fra expression initiates in developing neurons, including the earliest commissural axons, just prior to establishment of the axonal scaffold and is maintained during ventral nerve cord formation (Fig. 2B–D). Comparable fra expression patterns are detected in the developing nervous system of An. gambiae (Fig. 2A). These data are consistent with the hypothesis that Fra functions to regulate growth of commissural axons in mosquitoes.

si-RNA mediated knockdown of fra during Ae. aegypti development

Analysis of fra expression (Fig. 2) suggested that this gene may regulate ventral nerve cord development in mosquitoes. Functional testing of this hypothesis required the development of a strategy to selectively inhibit gene function during mosquito development. RNA interference (RNAi) technology, which has emerged as an effective method for inhibiting gene function in many organisms, was therefore combined with previously described Ae. aegypti microinjection techniques [30,39] to knockdown fra during Ae. aegypti development. Two separate siRNAs corresponding to

![Figure 1. Development of the Ae. aegypti embryonic ventral nerve cord](image-url)

**Figure 1. Development of the Ae. aegypti embryonic ventral nerve cord.** Anti-acetylated tubulin staining (A–C) marks the developing axon tracts in 52 hr. (A) and 56 hr. (B) Ae. aegypti embryos. By 56 hrs. (C), the Ae. aegypti nerve cord resembles that of a 33 hr. An. gambiae embryo and a St. 16 Dr. melanogaster nerve cord (BP102 staining is shown in D). These time points in the three respective species correspond to germ-band retracted embryos in which segmentation is obvious and organogenesis has initiated. Filleted nerve cords are oriented anterior up in all panels. The anterior commissure is marked by a black arrowhead, and a white arrowhead marks the posterior commissure.

doi:10.1371/journal.pone.0016730.g001
different regions of *Aae fra*, *fra* siRNA-A and *fra* siRNA-B, as well as a scrambled control version of *siRNA-A*, were used in these experiments.

*siRNAs* were injected pre-cellular blastoderm, and knockdown was assessed through both quantitative real-time PCR (qRT-PCR) and whole-mount *in situ* hybridization. Multiple qRT-PCR replicates at three different time points, including 24, 48 (not shown), and 72 hrs. (Fig. 3), confirmed knockdown of *fra* that was maintained through the end of embryogenesis. At 72 hrs., the time point that was typically assayed once injection protocols and knockdown strategies had been optimized, *fra* transcript levels were reduced by 80% on average (Fig. 3, *p* < 0.0001), and a maximum of 90% knockdown was achieved in one replicate. Knockdown in the developing CNS was verified through *in situ* hybridization, which confirmed reduced levels of *fra* transcripts in the embryonic CNS at levels comparable to those detected by qRT-PCR, and which revealed nearly complete knockdown in the developing nervous systems of embryos bearing strong phenotypes (Fig. 4C). These studies suggest that siRNA methodology can be used for targeted disruption of embryonic gene function in *Ae aegypti*.

*Ae. aegypti fra* knockdown CNS phenotypes

The impact of *fra* knockdown on *Ae. aegypti* embryonic nerve cord development was assessed through anti-acetylated tubulin staining at 54 hrs. AEL. In embryos injected with *fra* siRNA-A, 71% of anterior commissures and 80% of posterior commissures are thin or absent (Fig. 4B, C, Table 1). As observed in *Drosophila* [27], the posterior commissure is more severely disrupted than the anterior, with 51% of the embryos displaying a severe phenotype in the posterior commissure and 36% of embryos displaying a severe anterior commissure phenotype (Table 1). Occasional breaks in the longitudinal tracts were also noted in *fra* knockdown embryos. Injection of either *fra* siRNA-A (Fig. 4B,C) or *siRNA-B* (Fig. 4D), which correspond to two separate *Aae fra* sequences, produced similar phenotypes. This result indicates that the knockdown phenotypes described are due to loss of *fra* and are not the result of off-site targeting. Injection of the scrambled control *siRNA* did not disrupt nerve cord development (Fig. 4A, Table 1).

It should be noted that the penetrance and severity of the *Aae fra* knockdown phenotype are higher than that reported for the *Drosophila fra* null, in which only 12% of the anterior commissures and 43% of the posterior commissures are reportedly thin or absent [27]. In fact, in embryos in which CNS transcripts are nearly depleted, the *Aae fra* knockdown phenotype (Fig. 4B,C) bears strong resemblance to the *Drosophila commissureless* phenotype, in which commissure formation is entirely blocked [40]. These results suggest that Net-Fra signaling may play a more critical role in formation of the *Ae. aegypti* ventral nerve cord, and that the guidance cues postulated to compensate for loss of Net-Fra signaling in *Dr. melanogaster* [29] may not be present in mosquitoes.
These observations suggest that further analysis of embryonic nerve cord development in mosquitoes may uncover underlying differences between *D. melanogaster* and mosquito nervous system development. In support of this concept, our ongoing analysis of *senapthorin* knockdown in *Ae. aegypti* suggests that the function of this gene in nerve cord development has evolved in insects (data not shown).

### Developmental Genetics in Vector Mosquitoes

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. In this investigation, we examined the role of Fra during development of two vector mosquitoes. Expression of fra in the developing ventral nerve cord was found to be conserved between the two mosquitoes and *D. melanogaster*. However, the results of this investigation, the first targeted knockdown of a gene during vector mosquito embryogenesis, illustrate that although Fra plays a critical role during development of the *Ae. aegypti* ventral nerve cord, mechanisms regulating embryonic commissural axon guidance may have evolved in distantly related insects. This is a somewhat unexpected finding given the many similarities in insect CNS development that have been observed (for example, see [34,41]). Given these findings in *Ae. aegypti*, it would also be interesting to apply the siRNA-mediated knockdown strategies utilized here to *An. gambiae* and to formally assess the function of *Aga fra*.

Characterizing the function of additional developmental genes in mosquitoes is critical. To date, expression patterns of only a handful of mosquito developmental genes [9,10,11,12,13,14,15,16] have been reported. Adelman et al. [13] showed that control sequences for one of these genes, *nanos* [11], demonstrated promise as part of a transposable element-based gene drive system that may be used to spread and fix antipathogen effector genes in natural populations. Their investigations illustrate the exciting potential for the application of evo-devo methodologies used in this investigation, in particular the siRNA-mediated knockdown strategy for functional analysis of developmental genes in *Ae. aegypti* embryos, will broaden and enhance these efforts.

### Materials and Methods

#### Ethics statement

This study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was approved by the University of Notre Dame Institutional Animal Care and Use Committee (Study # 11-036).

#### Mosquito Rearing, Egg Collection, and Fixation

The *Ae. aegypti* Liverpool-IB12 (LVP-1B12) strain and *An. gambiae* [M Form] were used in these investigations. Procedures for mosquito rearing and egg collection [22,42], which was performed at 26°C, have been described. *Ae. aegypti* embryos were fixed as described [20]. *An. gambiae* embryos were fixed using a comparable procedure, except that eggs were fixed at room temperature.

#### Immunohistochemistry

Immunohistochemistry was performed as described [19]. Anti-acetylated tubulin (Zymed, San Francisco, CA) was used at a concentration of 1:100, and HRP-conjugated secondary antibod-
ies (Jackson ImmunoResearch, West Grove, PA) were used at a final concentration of 1:200.

**In situ hybridization**
Riboprobes corresponding to *Aae fra* (AAEI014592) and *Aae fra* (AGAP060083) were synthesized according to the Patel [43] protocol. In situ hybridization was performed as previously described [23].

**RNA interference**
 Knockdown was performed through embryonic microinjection of siRNAs targeting *Aae fra*. siRNA design and microinjection were performed as described [21]. The following siRNAs were synthesized by Dharmacon RNAi Technologies (Lafayette, CO): siRNA-A sense: CCA GAT GGG TAT GGG AGA T and antisense: GGG TCA CCC ATA GCC TCT A (corresponding to base pairs 3862–3883 of *Aae fra*). A scrambled version of siRNA-A was used as a control: sense GAT TAG ACG AAT ACC ACT A and antisense: CTA ATC TGC TTA TGG TGA T. siRNAs were injected at a concentration of 6 ng/ul.

Measurement of knockdown effectiveness was determined through *in situ* hybridization (see above) and through qRT-PCR. qRT-PCR was performed as previously described [44]. In short, total RNA was extracted from ~30 pooled siRNA-microinjected mosquito embryos using Trizol (Invitrogen, Carlsbad, CA). cDNA was prepared with the High Capacity RNA to cDNA Kit (Applied Biosystems, Foster City, CA), which includes a blend of random and oligo(dT) primers, according to the manufacturer’s instructions. Real-time quantification was performed using the SYBR Green I PCR kit (Applied Biosystems, Foster City, CA) in conjunction with an Applied Biosystems Step One Plus Real-Time PCR System. Primer sets for *Aae fra* were: For 5′ GCG ACC CAA CAC TCA ATA TG 3′ and Rev 5′ TTC GTG GTG ACC TGG ACA ACG ATG 3′. Three independent biological replicates were conducted, and all PCR reactions were performed in triplicate. Quantification of results was accomplished by standardizing reactions to *pB37* levels, and then using the ΔΔCt method as described [45]. Results were expressed as fold-difference compared with the scrambled control-injected embryos. qRT-PCR data from replicate experiments were statistically analyzed with the Student’s T Test.

**Acknowledgments**
We thank Frank Collins for advice, encouragement, and use of equipment. Many thanks to Nora Besansky and Marc Kern for *An. gambiae* eggs and to Nipam Patel for suggestions about fixing *An. gambiae* eggs. We are extremely grateful to Sun Longhua of the Malcolm Frazier lab who taught us to microinject mosquito embryos. William Browne suggested that we use siRNAs and gave expert advice on their design. Thanks to Becky DeBruyn and Phoebe Lovin for their technical assistance. We are grateful to the members of the Schell and Severson labs and the EIGH for their advice during the course of this investigation.

**Author Contributions**
Conceived and designed the experiments: AC DS MD-S. Performed the experiments: AC MH AM MT MD-S. Analyzed the data: AC MH MT DS MD-S. Contributed reagents/materials/analysis tools: AC AM MT MD-S. Wrote the paper: AC MH DS MD-S.

**References**
1. Holt RA, Subramanian GM, Häupler A, Sutton GG, Chard-R, et al. (2002) The genome sequence of the malaria mosquito *Anopheles gambiae*. Science 298: 129-149.
2. Nene V, Worwood JR, Lawson D, Haas B, Kodira C, et al. (2007) Genome sequence of *Aedes aegypti*, a major arbovirus vector. Science 316: 1718-1723.
3. Clemons A, Haugen M, Flannery E, Tiomek M, Kast K, et al. (2010) *Aedes aegypti*: an emerging model for vector mosquito development. Cold Spring Harb Protoc 2010: pdb emo11.
4. Chen XG, Mathur G, James AA (2008) Gene expression studies in mosquitoes. Adv Genet 64: 19–50.
5. Ramamani LN, Cupp, W E (1975) Early embryology of *Aedes aegypti* (Diptera: Culicidae): I. J Insect Morphol embryol 7: 273–296.
6. Farnesi LC, Martins AJ, Valle D, Rezende GL (2009) Embryonic development of *Aedes aegypti* (Diptera: Culicidae): organogenesis. Int J Insect Morphol Embryol 7: 275–286.
7. Farnesi LC, Martins AJ, Valle D, Rezende GL (2009) Embryonic development of *Aedes aegypti* (Diptera: Culicidae): influence of different constant temperatures. Mem Inst Oswaldo Cruz 104: 124–126.
8. Vital W, Rezende GL, Abreu L, Moraes J, Lemos IJ, et al. (2010) Geen hand retraction as a landmark in glucose metabolism during *Aedes aegypti* embryogenesis. BMC Dev Biol 10: 25.
9. Golob Y, Hsiung W, Lanzaro G, Levine M (2004) Different combinations of gap exressors for common stripes in *Anopheles* and *Drosophila* embryos. Dev Biol 275: 435–446.
10. Calvo E, Walter M, AdelmanZN, Jimenez A, Oral S, et al. (2005) Nason (noo) genes of the vector mosquitoes, *Anopheles gambiae*, *Anopheles stephensi* and *Aedes aegypti*. Insect Biochem Mol Biol 35: 789–798.
11. John J, James AA (2006) oskar gene expression in the vector mosquitoes, *Anopheles gambiae* and *Aedes aegypti*. Insect Mol Biol 15: 363–372.
12. John J, Marinotti O, Calvo E, James AA (2008) Gene structure and expression of nanos (nos) and oskar (osk) orthologues of the vector mosquito, *Caesio cyanophrys*. Insect Mol Biol 17: 545–552.
13. Adelman ZN, Jasinskiene N, Oral S, John J, Ashiyaka A, et al. (2007) nason gene control DNA mediates developmentally regulated transposition in the vector mosquitoes, *Anopheles gambiae*, *Aedes aegypti*. Insect Biochem Mol Biol 35: 789–798.
14. Golob Y, Fuse N, Frasch M, Zinners R, Lanzaro G, et al. (2007) Evolution of the dorsal-ventral patterning network in the mosquito, *Anopheles gambiae*. Development 134: 2415–2424.
15. Golob Y, Rezende GL, Franizan K, Lanzaro G, Valerie D, et al. (2009) Developmental and evolutionary basis for drought tolerance of the *Anopheles gambiae* embryo. Dev Biol 330: 462–470.
16. Simanton W, Clark S, Clemons A, Jacowski C, FarrellVanZomermeren, A, et al. (2009) Conservation of arthropod midline netrin accumulation revealed with a cross-reactive antibody provides evidence for midline cell homology. Evol Dev 11: 260–268.
17. Christophers SR (1960) *Aedes aegypti*, The yellow fever mosquito: its life history, bionomics, and structure. Cambridge, UK: Cambridge University Press.
18. Severson DW, DeBruyn A, Leaon DD, Browne SE, Krondon DL, et al. (2004) Comparative genome analysis of the yellow fever mosquito, *Aedes aegypti* with *Drosophila melanogaster* and the malaria vector mosquito *Anopheles gambiae*. J Hered 95: 103–113.
19. Clemons A, Flannery E, Kast K, Severson D, Duman-Scheel M (2010) Immunohistochemical analysis of protein expression during *Aedes aegypti* development. Cold Spring Harb Protoc 2010: pdb prot5510.
20. Clemons A, Haugen M, Kast K, Jacowski C, et al. (2010) Fixation and preparation of developing tissues from *Aedes aegypti*. Cold Spring Harb Protoc 2010: pdb prot5511.
21. Clemons, A, Hauguen M, Severson K, Duman-Scheel M (2010) Metanephric development of *Aedes aegypti* embryos. Cold Spring Harb Protoc 2010: pdb prot5507.
22. Clemons, A, Mori A, Hauguen M, Severson DW, Duman-Scheel M (2010) Functional analysis of genes in *Aedes aegypti* embryos. Cold Spring Harb Protoc 2010: pdb prot5507.
23. Clemons, A, Mori A, Hauguen M, Severson DW, Duman-Scheel M (2010) Metanephric development of *Aedes aegypti* embryos. Cold Spring Harb Protoc 2010: pdb prot5507.
24. Clemons, A, Mori A, Hauguen M, Severson DW, Duman-Scheel M (2010) Metanephric development of *Aedes aegypti* embryos. Cold Spring Harb Protoc 2010: pdb prot5507.
25. Kaprielian Z, Runko E, Imondi R (2001) Axon guidance at the midline choice point. Dev Dyn 221: 154–181.
26. Duman-Scheel M (2009) Nettin and DCC: axon guidance regulators at the intersection of nervous system development and cancer. Curr Drug Targets 10: 275–289.
27. Kołodziej PA, Timpe LC, Mitchell KJ, Fried SR, Goodman CS, et al. (1996) Frazzled encodes a *Drosophila* member of the DCC immunoglobulin subfamily and is required for CNS and motor axon guidance. Cell 87: 197–204.
28. Hiramoto M, Hiromi Y, Giniger E, Hotta Y (2000) The Drosophila netrin receptor frazzled guides axons by controlling netrin distribution. Nature 406: 886–889.
29. Harris R, Sahatçulli LM, Seeger MA (1996) Guidance cues at the Drosophila CNS midline: identification and characterization of two Drosophila Netrin/UNC-6 homologs. Neuron 17: 217–228.
30. Mitchell KJ, Doyle JL, Serafini T, Kemedy TE, Tessier-Lavigne M, et al. (1996) Genetic analysis of netrin genes in Drosophila: netrins guide CNS commissural axons and peripheral motor axons. Neuron 17: 203–215.
31. Brankatschk M, Dickson BJ (2006) Netrins guide Drosophila commissural axons at short range. Nat Neurosci 9: 188–194.
32. Gerberding M, Scholtz G (1999) Cell lineage of the midline cells in the amphipod crustacean Orchestia cavimana (Crustacea, Malacostraca) during formation and separation of the germ band. Dev Genes Evol 209: 91–102.
33. Gerberding M, Scholtz G (2001) Neurons and glia in the midline of the higher crustacean Orchestia cavimana are generated via an invariant cell lineage that comprises a median neuroblast and glial progenitors. Dev Biol 235: 397–409.
34. Duman-Scheel M, Patel NH (1999) Analysis of molecular marker expression reveals neuronal homology in distantly related arthropods. Development 126: 2327–2334.
35. Browne WE, Schmid BG, Wimmer EA, Martindale MQ (2006) Expression of otd orthologs in the amphipod crustacean, Parhyale hawaiensis. Dev Genes Evol 216: 581–595.
36. Duman-Scheel M, Clark SM, Grunow ET, Hasley AO, Hill BL, et al. (2007) Delayed onset of midline netrin expression in Artemia franciscana coincides with commissural axon growth and provides evidence for homology of midline cells in distantly related arthropods. Evol Dev 9: 131–140.
37. Doe CQ, Goodman C (1993) Embryonic development of the Drosophila central nervous system. In: Martinez Arias A, ed. The Development of Drosophila melanogaster. Plainview, NY: Cold Spring Harbor Laboratory Press. pp 1091–1130.
38. Lobo NF, Clayton JR, Fraser MJ, Kafatos FC, Collins FH (2006) High efficiency germ-line transformation of mosquitoes. Nat Protoc 1: 1312–1317.
39. Jasińskiene N, Juht J, James A (2007) Microinjection of A. aegypti embryos to obtain transgenic mosquitoes. Journal of Visualized Experiments 5.
40. Seeger M, T ear G, Ferrer-Marco D, Goodman CS (1993) Mutations affecting growth cone guidance in Drosophila genes necessary for guidance toward or away from the midline. Neuron 10: 409–426.
41. Thomas JB, Bastiani MJ, Rate M, Goodman CS (1984) From grasshopper to Drosophila: a common plan for neuronal development. Nature 310: 203–207.
42. Benedict MQ (1997) The Molecular Biology of Insect Vectors of Disease. In: Crampton JM, Beard CB, Louis C, eds. London: Chapman and Hall. pp 3–12.
43. Patel N (1996) In situ hybridization to whole mount Drosophila embryos; Krieg PA, ed. New York: Wiley-Liss. pp 357–370.
44. Morlais I, Mori A, Schneider JR, Severson DW (2003) A targeted approach to the identification of candidate genes determining susceptibility to Plasmodium gallinaceum in Aedes aegypti. Mol Genet Genomics 269: 753–764.
45. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402–408.