

Transcriptional Analysis of Four Family 4 P450s in a Puerto Rico Strain of *Aedes aegypti* (Diptera: Culicidae) Compared With an Orlando Strain and Their Possible Functional Roles in Permethrin Resistance

WILLIAM R. REID,¹ ANNE THORNTON,^{2,3} JULIA W. PRIDGEON,^{4,5,6} JAMES J. BECNEL,⁴ FANG TANG,^{1,7} ALDEN ESTEP,^{2,4} GARY G. CLARK,⁴ SANDRA ALLAN,⁴ AND NANNAN LIU¹

J. Med. Entomol. 51(3): 605–615 (2014); DOI: <http://dx.doi.org/10.1603/ME13228>

ABSTRACT A field strain of *Aedes aegypti* (L.) was collected from Puerto Rico in October 2008. Based on LD₅₀ values by topical application, the Puerto Rico strain was 73-fold resistant to permethrin compared with a susceptible Orlando strain. In the presence of piperonyl butoxide, the resistance of Puerto Rico strain of *Ae. aegypti* was reduced to 15-fold, suggesting that cytochrome P450-mediated detoxification is involved in the resistance of the Puerto Rico strain to permethrin. To determine the cytochrome P450s that might play a role in the resistance to permethrin, the transcriptional levels of 164 cytochrome P450 genes in the Puerto Rico strain were compared with that in the Orlando strain. Of the 164 cytochrome P450s, 33 were significantly ($P < 0.05$) up-regulated, including cytochrome P450s in families four, six, and nine. Multiple studies have investigated the functionality of family six and nine cytochrome P450s, therefore, we focused on the up-regulated family 4 cytochrome P450s. To determine whether up-regulation of the four cytochrome P450s had any functional role in permethrin resistance, transgenic *Drosophila melanogaster* Meigen lines overexpressing the four family 4 P450 genes were generated, and their ability to survive exposure to permethrin was evaluated. When exposed to 5 μ g per vial permethrin, transgenic *D. melanogaster* expressing *CYP4D24*, *CYP4H29*, *CYP4J15v1*, and *CYP4H33* had a survival rate of 60.0 ± 6.7 , 29.0 ± 4.4 , 64.4 ± 9.7 , and $11.0 \pm 4.4\%$, respectively. However, none of the control flies survived the permethrin exposure at the same concentration. Similarly, none of the transgenic *D. melanogaster* expressing *CYP4J15v1* or *CYP4H33* ≥ 5 survived when they were exposed to permethrin at 10 μ g per vial. However, transgenic *D. melanogaster* expressing *CYP4D24* and *CYP4H29* had a survival rate of 37.8 ± 4.4 and $2.2 \pm 2.2\%$, respectively. Taken together, our results suggest that *CYP4D24* might play an important role in cytochrome P450-mediated resistance to permethrin.

KEY WORDS *Aedes aegypti*, permethrin, resistance, cytochrome P450, detoxification

The yellowfever mosquito, *Aedes aegypti* (L.), is a globally distributed mosquito and the major vector of dengue virus (Gubler 1988, Warren and Mahmoud 1990, Gubler and Clark 1995). Management of *Ae. aegypti* is primarily through the use of chemical insecticides, of which pyrethroids were frequently used

because of their low mammalian toxicity and high efficacy (Hougard et al. 2002, Juntarajumnong et al. 2012, Manda et al. 2013). However, frequent use of insecticides has led to the development of insecticide resistance in field mosquitoes (Hemingway et al. 2004, Ffrench-Constant et al. 2004, Liu 2008), making the management of *Ae. aegypti* populations problematic as higher doses of insecticide are needed to obtain the same level of control, ultimately leading to control failure (Vulule et al. 1994, Curtis et al. 1998, Liu 2008).

Insecticide resistance is a multifaceted phenomenon involving several mechanisms, including target site insensitivity, reduced penetration rate, and metabolic detoxification. In the case of metabolic detoxification, three major classes of enzymes are involved: cytochrome P450s, hydrolases, and glutathione-S-transferases (Feyereisen 1995, Ffrench-Constant et al. 2004, Hemingway et al. 2004, Yang and Liu 2011, Reid et al. 2012, Gong et al. 2013). Of the three detoxification enzymes, the role of cytochrome P450s in *Ae.*

¹ Department of Entomology and Plant Pathology, Auburn University, 350 South College Street, Auburn, AL 36849.

² Navy Entomology Center of Excellence, 937 Child Street, Jacksonville, FL, 32312.

³ Current address: Department of Medicine and Department of Genome Sciences, University of Washington, Foege Building S-250, Box 355065, 3720 15th Ave., NE, Seattle WA 98195-5065.

⁴ USDA-ARS, Mosquito and Fly Research Unit, Center for Medical, Agricultural, and Veterinary Entomology, 1600 SW23rd Dr., Gainesville, FL 32682.

⁵ Current address: USDA, ARS, Aquatic Animal Health Research Unit, 990 Wire Rd., Auburn, AL 36832.

⁶ Corresponding author, e-mail: Julia.pridgeon@ars.usda.gov.

⁷ Current address: Institute of Vegetables and Flowers, Chinese Academy of Agricultural Science, 12 Zhongguancun Nandajie, Beijing 100081, China.

Report Documentation Page		Form Approved OMB No. 0704-0188
Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.		
1. REPORT DATE 2014	2. REPORT TYPE	3. DATES COVERED 00-00-2014 to 00-00-2014
4. TITLE AND SUBTITLE Transcriptional Analysis of Four Family 4 P450s in a Puerto Rico Strain of Aedes aegypti (Diptera: Culicidae) Compared With an Orlando Strain and Their Possible Functional Roles in Permethrin Resistance		5a. CONTRACT NUMBER
		5b. GRANT NUMBER
		5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)		5d. PROJECT NUMBER
		5e. TASK NUMBER
		5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Navy Entomology Center of Excellence,937 Child Street,Jacksonville,FL,32312		8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)		10. SPONSOR/MONITOR'S ACRONYM(S)
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution unlimited		
13. SUPPLEMENTARY NOTES		
14. ABSTRACT A ?eld strain of Aedes aegypti (L.) was collected from Puerto Rico in October 2008. Based onLD50 values by topical application, the Puerto Rico strain was 73-fold resistant to permethrin compared with a susceptible Orlando strain. In the presence of piperonyl butoxide, the resistance of Puerto Rico strain of Ae. aegypti was reduced to 15-fold, suggesting that cytochrome P450-mediated detoxi?cation is involved in the resistance of the Puerto Rico strain to permethrin. To determine the cytochrome P450s that might play a role in the resistance to permethrin, the transcriptional levels of 164 cytochrome P450 genes in the Puerto Rico strain were compared with that in the Orlando strain. Of the 164 cytochrome P450s, 33 were signi?cantly (P 0.05) up-regulated, including cytochrome P450s in families four, six, and nine. Multiple studies have investigated the functionality of family six and nine cytochrome P450s, therefore, we focused on the up-regulated family 4 cytochrome P450s. To determine whether up-regulation of the four cytochrome P450s had any functional role in permethrin resistance, transgenic Drosophila melanogaster Meigen lines overexpressing the four family 4 P450 genes were generated, and their ability to survive exposure to permethrin was evaluated. When exposed to 5 g per vial permethrin, transgenic D. melanogaster expressing CYP4D24, CYP4H29 CYP4J15v1, and CYP4H33 had a survival rate of 60.0 6.7, 29.0 4.4, 64.4 9.7, and 11.0 4.4% respectively. However, none of the control ?ies survived the permethrin exposure at the same concentration. Similarly, none of the transgenic D. melanogaster expressing CYP4J15v1 or CYP4H33 ?5 survived when they were exposed to permethrin at 10 g per vial. However, transgenic D. melanogaster expressing CYP4D24 and CYP4H29 had a survival rate of 37.8 4.4 and 2.2 2.2% respectively. Taken together, our results suggest that CYP4D24 might play an important role in cytochrome P450-mediated resistance to permethrin.		
15. SUBJECT TERMS		

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Same as Report (SAR)	18. NUMBER OF PAGES 11	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			

Table 1. List of primers used for the qPCR and the generation of constructs for the functional testing in transgenic *D. melanogaster*

Primer	Gene ^a	Vectorbase ^b	Forward primer (5'-3')	Reverse primer (5'-3')
qPCR	CYP15B1	AAEL002067	CGGATTTCGTTCTTCGATAA	ATGGAATTCAGCACCGAAAC
	CYP18A1	AAEL004870	CAGTGAAGGTCAGCTGTGGA	CGAGACGGAGAGCTACTTGC
	CYP303A1ae	AAEL012144	GATAGCACGAGCAGACAAA	CCAAGTCGGGGTTTCATAGA
	CYP304B2xx/yy	AAEL014412	GATTGGAAGGAGCAGAGACG	CCTTTACCGGTTAGACAT
	CYP304B3yy/xx	AAEL014411	GGTCAGTTCTACCGGACCAA	TCAATGCCTCAGCACAAAG
	CYP304C1	AAEL014413	GGGAGAAATCTACCGGAAAGG	CTCGCGGTACATTTTGGTTT
	CYP305A6	AAEL002071	GCTCCCATCTTCGGTAACA	TTCCCAATCTGGTTCGCATA
	CYP305A5	AAEL002043	AGCCCTCTCCAAGCAGTACA	AGCCTTGTCCCCATAGTCTCT
	CYP306A1	AAEL004888	TCGTGTGATATCCGCATGT	GGGAGGTAGTACAGCGTTTC
	CYP307A1	AAEL009762	GCCCTGCTGAAAAGTCTACG	GCCTTGTTCGGTAACGAT
	CYP307A1	AAEL009768	GAGTCAGCCTCAGGAAGTGG	CCCGTTCTGATTCAACACCT
	CYP307B1	AAEL006875	ATCATGGAAGCGCTGAGACT	GGTTTCTCCAGAGGTTCTCC
	CYP6F2	AAEL014678	CGTGAGTGCACCCAAGACTA	GAGCGTACGGTTTGTCTTTC
	CYP6F3	AAEL014684	GTGGCGTTTGGCATTAAAGAT	CCGTACGACACCGTTTTTTCT
	CYP6M5	AAEL009117	TGGATCTGCTGATCGGTATG	AGCACTTCTTGACGCGATTTC
	CYP6M6	AAEL009128	AGAAAAATACCAACCGTTCC	GGTCAATCTTTTCCGATCA
	CYP6M10	AAEL009125	TGAGTCAAACTCGATGAAGG	ATTCCACCGTGTCTTTTACG
	CYP6M11	AAEL009127	TTGTTACAGCAGGCAGAAAG	CCTCGTGTCTTTTATTTCTCG
	CYP6N6	AAEL009126	TTCTTCATCGGTGCTGTGAG	TTTTCGGCAAGTTCAGTACAAG
	CYP6N9	AAEL009121	ACCGCAACCCAAGACTACAC	AAACGCATCCCGATACAGAC
	CYP6N11	AAEL009119	CTGCCCTGTCTACGCAATTCA	CAAAATTTTCAACGGGCTTG
	CYP6N11	AAEL009138	TGGGTTATCTGGCGCTATTTC	ACACATCTTGGCCAGGTTCC
	CYP6N12	AAEL009124	TTCACTTGGCGGATCACTAC	TGCAGCAATTTCTCAACAG
	CYP6N13	AAEL009137	ACAAATGTCCCGAACTCGAAC	CATCATTTCCGAATCGTGTG
	CYP6N14	AAEL009133	ACGTTTCTTCCGGGAACT	TTTCGCCCATTTTCTGAAAC
	CYP6N15	AAEL009122	GTCAAGGCGTACCGTTTCACT	CGCGATCGTGAAGTACTGA
	CYP6N16	AAEL010151	AAAACCTCGCAAGAAAAGCA	GCCACCACTCGTTTCATACT
	CYP6N17	AAEL010158	AAGCATCACCCAGAACCAAC	ACCTTCTTGGCCAGGTTCTT
	CYP6P12v1	AAEL012491	GGCAGTTTTGGTGGACAGT	CTGCTCGAACACGTTTCTTCC
	CYP6S3	AAEL009120	AGGCAGATGGGGAAGAGAAT	TCAACAGCTGCATGAAGTCC
	CYP6Y3	AAEL009132	GCTAGTGGCTGCCGTCTTAC	CAGAGCGCAAGTCAACATGA
	CYP6Z6	AAEL009123	CGAGGTGTCTACTGCAACGA	TAACCTGTGCCAACATCCA
	CYP6Z7	AAEL009130	GAGATCCGTTTTCTGCAAGC	GGTTCCGGATTCTCAGCTAC
	CYP6Z8	AAEL009131	CCTGAGATGATCCGATTCTGT	CTCTTTCGAAACCCCAAGCTG
	CYP6Z9	AAEL009129	TCCAATGGAGCAATCACGTA	ATCGTTCGGGAATGTAGCAC
	CYP6AA5v1	AAEL012492	CCAGCTTCGAGCCTTTTATG	TGAAGACTCTGTCCGCAATG
	CYP6AC3	AAEL007024	CCGAACGTTTTTAACCCAGAA	CTGCTTTCGGCAAAAGTAGC
	CYP6AC5	AAEL006984	ACATTCCGCAGAAAGATGGTC	TAGGTGGATAGCCAGATCG
	CYP6AC6	AAEL006992	ACACCCGCGTTTACTACGTC	GGCGATCAACAAGCAAGATA
	CYP6AC7	AAEL006989	TGTTTCCAGCTGCATCTTTG	TTTCGCTTGCATCAGATAG
	CYP6AC8	AAEL003890	ACCAGCACACGGAAGTACC	AACCTGCATACGACCGAAAC
	CYP6AH1	AAEL007473	CTGCGTGTGAAAAGACTACG	ATCCGCTTACCAACGTCATC
	CYP6AH1	AAEL015641	CTGCGTGTGAAAAGACTACG	ATCCGCTTACCAACGTCATC
	CYP6AK1	AAEL004941	AAGGATTGTACGCCGATTTC	CTCAAGGAATCCGGTTACGA
	CYP6AL1	AAEL008889	AACCGAGAATGCACCAAAAC	CGACTGTTCGTCATCTGGAGA
	CYP6AL3	AAEL009656	GGCAAAAGCTTTCATCAGGAAA	CACATAGGTTGTGCCCTTCT
	CYP6BB2	AAEL014893	TAGTCGCTAAGGACGCGAGGA	AAGTACTCCGGATCGTGGTG
	CYP6BZ1	AAEL012494	GTAGGGCAAATGCTGTGGAT	ACTCCGTTGAACGTTGTTC
	CYP6CA1	AAEL014680	TCGAGGCACTTTCAGCACTT	AACAAATCGGCCACGCTAC
	CYP6CB1	AAEL002046	TAAGCAGCGCACCTCCTATT	AAAATCAACCGTACAGCATCC
	CYP6CB1	AAEL009018	GAGTGCAACAGCATGAGGAA	GTCATCGTCTGATCAGCTTCA
	CYP6CB2	AAEL002872	GTTTCGGAGATGGTCCAAAGA	GGTTGAAGCATCAGCATGA
	CYP6CC1v1	AAEL014890	GGGAACAGTTGGCAGGATAA	AAGTCGCTGTTTGGGCTTGT
	CYP6CD1	AAEL005006	TGGCCATTCTCTAGCGTTCT	TGCAGGTGGTGTATCCGTA
	CYP9J2	AAEL006805	ACCGTTACGCCAACAAAGAC	GACGATTTTTCGATCGGTTGT
	CYP9J6	AAEL002638	CAGCGTCAAACCAAGGGTAT	GTTTCAAATCCAGCCAGGAA
	CYP9J7	AAEL014606	CGGATATGGTGCATCTGTTG	GGTTCAACGCCAGTTCGAT
	CYP9J8	AAEL006811	CCTCAACCGCAAGTACCAAT	GTCTTGCACCCGATTGCT
	CYP9J9	AAEL006793	TGATCGCTCAGTGTTCCTG	TTCCGTAGGTCAATGGCTTCC
	CYP9J9	AAEL014605	TGATCGCTCAGTGTTCCTG	TTCCGTAGGTCAATGGCTTCC
	CYP9J10	AAEL006798	TATGGCGGAGTTTTTCAAGG	CACCGATAGCGATTGGAAGT
	CYP9J15	AAEL006795	GTACTACCCACAGCCGGAAG	ACACACCGCTTTTCATCTCC
	CYP9J16	AAEL006815	ACGATTGCCATACCAACGA	TTCGCTCTTCTCCGTAGGT
	CYP9J17	AAEL009699	GGAGAAATTCGGGGTTGATT	TCATCCATCTCGTGCTTCAG
	CYP9J17	AAEL006784	TAAAGGCGAGCACTGAAGCAC	AGGTCAAACCCGTAGACACG
	CYP9J18v1	AAEL006804	GCCAGATCCAGATCGTTTC	AATTTCGCTCTTTTCGGTTG
	CYP9J19	AAEL006810	CCAACCTTTCTCGTTGGAAG	CTTCTACGGGTTGGTCCGTA
	CYP9J19	AAEL014611	CATCCAGAACGATCCGAACT	GTCCGCTCCAGACTGAACTC
	CYP9J20v1	AAEL006814	ACGGAACGACATGATCAACA	AGCACTCGTAGGCCAAGAA
	CYP9J20v2	AAEL014604	TGAGCTTGATGTTCCAGCTG	CCCATTGGTGAAGAACTCGT
	CYP9J21	AAEL014612	GTACAGCGTCTTTCGGAAGC	TGCATCAACAGGTGGATCAT

Continued on following page

Table 1. Continued

Primer	Gene ^a	Vectorbase ^b	Forward primer (5'-3')	Reverse primer (5'-3')
CYP9J22		AAEL006802	TGTTGATGCAGGCAAGAAG	CTGCCAGCAAAAACAGCAAG
CYP9J23		AAEL014615	GTGCCACCTTTCCGGAGTTA	AAGGGTCTTCTCGAACACGA
CYP9J24		AAEL014613	TTCCCAACGTATGCGTTACA	GAGGAACCTCCGTCTTGCTG
CYP9J26		AAEL014609	AGATGATCGCACAGTGCCTTG	GGGCCACATTCTCAAGTGT
CYP9J27		AAEL014616	ACGGCAAGAAAAATGATGGAC	CGGTTCCATGACTCTCCCTA
CYP9J27		AAEL014607	CGGCACGTAAGAAAATGAT	AGCCTTGATCGTCTCCTGAA
CYP9J28		AAEL014617	TTTCTCGACAAACCGATTTC	AAAGTCCTTAAACGGCCACCT
CYP9J29		AAEL014610	GATGACGACGACAGGTCAAA	AGTGGATTGCCCATTTTGAAG
CYP9J30		AAEL014603	TCAAAGTCCTCGGGATGTTC	ATATTACGCCATCGCTGACC
CYP9J31		AAEL002633	TTTTACGCGATTGAGTGTCC	ATATCCTTGGTCTGGCGTTG
CYP9J32		AAEL008846	GCCGTGACTACGTTTTTGAT	CTCCGATCCCAATGCAATTCCT
CYP9M4		AAEL001320	GGTTGATCAGCAAGGACGTT	AACCGTCGTAAATCCAGCAC
CYP9M5		AAEL001288	GTCCGTTCTCAGCTTCCGTTT	ATGGTCCTCGAACGCTGTAGG
CYP9M6		AAEL001312	CAGTGGCCACCTACGATTTT	CTCCGATCCGTCGATAAGAT
CYP9M7		AAEL001292	AGGACTATGGCCGGTTTTCT	GCCCTAGAATCGGATCAACA
CYP9M8		AAEL009591	AGCTGCCACTGTAGCCACTT	TTTGGTCGATCGATTCCGTAG
CYP9M9		AAEL001807	CACATAAGGAAATGGCCCTCA	TTCCGATCAAAATCTTTCTGG
CYP9AE1		AAEL003748	CAGTTCGGGATGAGTTGTT	TCAACTCGTCGTCACCTCAG
CYP329B1		AAEL003763	CTTCTGGACGAAAAGCAAAG	TAGTTCCGAATCCACGGAAAG
CYP4C38		AAEL012266	GAAAAAGTCCACCGCTATGA	CTTCGTCATGAAGGGGAAA
CYP4C50		AAEL008017	AAATTCCGGAAGCAGAAGCAA	ATGCCCTCGATCAACAGATCC
CYP4C51		AAEL008018	CAATCGACAAGCTCAGACCA	GCAATTATCCGTCGGATTCTT
CYP4C52		AAEL008023	TTCTCTCGATCGGGTCATTAG	CTTTTCTCCACCGAGCTTTG
CYP4D23		AAEL007816	GTTCACAAAGCCGAAGATCA	TTTATCGGAGTTCCCATTCG
CYP4D24		AAEL007815	TACTTCACCCCGTACCGAAG	AGGGGTCTCCCGTCTACTGT
CYP4D37		AAEL007795	GGAGACCGCTTTGCTTCTGAG	CAAGGCTCAGCAGACACAT
CYP4D38		AAEL007807	CAAGCAACCCGATGAATTTT	CGAGCCAGTTGGAGAGGTAG
CYP4D39		AAEL007808	CGTCGACGCAATAAACTCA	CCTCTCGATCGTGAGGAAAA
CYP4G35		AAEL008345	GGACCGATGGCTTCAGAAATA	GCATCAAGCAAAAGGCAACA
CYP4G35		AAEL006824	GGTCGTCAAGCAGAAAGAAG	GAAATCCAGATCGTCCCTGA
CYP4G36		AAEL004054	AACACCAATAGCGTCAAGG	CCCATCATCGAAAAGGAAGAA
CYP4H28		AAEL003380	ACACCGAAGGTGAAACCAAG	GGGCCATCAACGAGAGATAA
CYP4H29		AAEL007830	TGCAGGCTGTCAAAGAAATG	GATTCTTGCCCTTGCTTGCTC
CYP4H30		AAEL003399	GCTGCTGAAAAATAGCGAACC	GTCCCCCAGGAATAGGACAT
CYP4H31		AAEL002085	ACAAATTGCTGACGGTGTTC	TGGATTCTCTCGGGCATTC
CYP4H32		AAEL007812	ATCCAAATGCTTGGGAACAG	CTTCTCTCGGATGTCTTCC
CYP4H33		AAEL013798	CAGTAATTGATGCGCGAAGA	ATGACCCTCGAACATGAAGG
CYP4J13		AAEL013555	CAGGACCGTTGGAAGTTGAT	AGAGGCCACAGTACCGTTTT
CYP4J14		AAEL013554	AAGTTGTCCACGGTTTTCTGG	GAACGAATGAACCGGAAGAA
CYP4J15v1		AAEL013556	GCTGGATTGCTTTTACTCG	GAAAGCTCGGGATCACTGAG
CYP4J15v2		AAEL014829	AAACATCGATGGGCGTTAAG	AGCCGCTTTATAGCCTGTCA
CYP4J16		AAEL015663	AACGGATCATGAACCTCTG	TTCCGCCAGCAGAAAGACTAT
CYP4J17		AAEL015370	AGAATTCCCTACCGCTTGT	GGCCAGTAAGGTATCCAGCA
CYP4J17		AAEL014019	GGAGGAGATCGAAAACCATGA	ACTGTGCATCTCCAAAACC
CYP4K3		AAEL007798	GGAAGTGCAACGGAATTTGT	CTTCCGACTTTGAGGCGTAG
CYP4AR2		AAEL010154	CGGAGGTTTGCAATGATTCT	CGCTGGGTACTTTGCCATT
CYP325E3		AAEL000338	CAATAGGCTGTTTCGGGAGA	CTGTTAAGCATCGGGGTGTT
CYP325G2		AAEL012766	CTTATCGGTTGTGGCCATCT	CTGCATATCTTCCGGGTGT
CYP325G3		AAEL012772	TACCCTTTGATCGGAAGTGC	ACCGGTGAAGTGAACAGTCC
CYP325K2		AAEL005771	ATTTTGGCCGCTATCTTCCT	TTCTCTGGCAATAGGGATG
CYP325K3		AAEL005788	ATTCCGAAGGGAACGTCTCT	CAAGTCGCTGCTGAGTTCAA
CYP325L1v1		AAEL011770	TCGGTGGAACGAAACTACC	GTTCCGTCCGAGGATGTTGT
CYP325M1		AAEL012773	AGACGAAAAGTTCCGACGAT	CCTCTTGATAGCAGCGTTCC
CYP325M2		AAEL012769	TTGCTTCTTTGGGTTCCAC	TGCTGCTTTCCAAACGTATTG
CYP325M2		AAEL012772	TTCTGTTCTTTGGGTTCCAC	TGCTGCTTTCCAAACGTATTG
CYP325M3		AAEL012765	CGATCTGTGTGGAGAGCAAA	GTTTTCCGCGTTGTTTCATT
CYP325M4		AAEL011769	CGTTGAATCCTTCTGTTTGGT	GGCCGTTGCATACATTGAT
CYP325M5		AAEL011761	TGGAATAATCAACGGAAGC	TCGCTGCATATCGAAGTCAC
CYP325N1		AAEL012770	GTACCTTGAAGCGCAAGAGG	TGTTACGATCTCTTGCTTG
CYP325N2		AAEL012762	CTTGCCCGGATAGAAATCAA	TCCGGGTGAAAGTTTTGAC
CYP325P1		AAEL000340	CGTGGTTGATTTCGGAGTTT	GCCTGGGTGTGATTTTCTGTT
CYP325Q1		AAEL006044	ACCACGGAAGCTCTGAAAAA	CATCTGCTCCCATACATCC
CYP325Q2		AAEL015563	ACGAAAGTCGGGAAGAAGA	CAGGTGTAGGAAACGGCATT
CYP325R1		AAEL005775	CGGCTTACTCATGGGTTGTT	TATCACTGGAGTCCCTTCG
CYP325S1		AAEL000326	CCGATTTTCTTCGACAGCTC	GCAAAATTCTCCGGATCAAA
CYP325S2		AAEL000325	GCCGAAATCATGGAACACTT	CCCATATCCGCTCTTCGAT
CYP325S3		AAEL000357	TTGCTCGGCAGTGATCAAG	TCCGGATAGGAAATGTCTGG
CYP325T2		AAEL012761	GAGTTTGCCATCCGATCTA	CCTGCTGCAACACTTTTCAA
CYP325T2		AAEL015475	CTCATGGCCTATGCCTGTTT	GCCATGTTTTGCCTTACGAT
CYP325U1		AAEL000320	GGGAAAAAATGCTGAGGAAT	ATTCAGTCCCTTACCGTGCT
CYP325X1		AAEL005695	CTGTACCGGTTTGCTGGATT	TCGTCATCTTCTCGAACAC

Continued on following page

Table 1. Continued

Primer	Gene ^a	Vectorbase ^b	Forward primer (5'-3')	Reverse primer (5'-3')
Drosophila constructs	CYP325X2	AAEL005696	TGTCGTTCTACCCGGAAATC	TGCAACTCGGCCATATTTTC
	CYP325X4	AAEL005700	GGCTCAACTCCAAGCTTCAAC	CGAATTCTCTTTCCCTTCC
	CYP325Y1	AAEL006257	GAAATCGTGCTCGATGGAAT	AGATAGGCAAAATGGGTGACC
	CYP325Y2v2	AAEL015362	AGGAAGCCCTCCGTATTTGT	ATGCCCTTTGCTGAACTGCTT
	CYP325Y3	AAEL006246	GGCATAACGCATCCCTAAAGA	TCTGCATAATCCGCAACAAA
	CYP325Y3	AAEL015361	CAATCGCTTGGTGAGGATTT	CCTCCGGGTACATTGCTAGA
	CYP325Z1	AAEL010273	CACCAAATCCAAGCCAAACT	GTCTTTCCGCCCTGTGAAGAG
	CYP325AA1	AAEL004012	TGCTTTCTGTCGATCGTAGTG	CACCAGCTCTGGATGCTGTA
	CYP12F5	AAEL001960	ACAAGGAGAAAGCTGGCAAA	CATCGAGAACTCCCAATCGT
	CYP12F6	AAEL002005	TACATCGTTGACTCCGGACA	CGAAGCGATCACTTTTGTGA
	CYP12F7	AAEL002031	CTGGAACCGATCGGTGTTCT	GATAACCGCGTCATCAACCT
	CYP12F8	AAEL006827	TGGATAAGGTTGCCCTTCAG	TCTCCAGATCGAGGGAAGAA
	CYP49A1	AAEL008638	GTGCATCAAAGAAACGCTGA	CGGTCTGGTTCTGGGAAATA
	CYP301A1	AAEL014594	CCTGGAACCGAACTTGACAT	GTCCTTCAACCAAGTTTCAT
	CYP302A1v1	AAEL011463	TTTCGATGTACGGTTGGACA	GCTTTTCGATACGCTGGAGTC
	CYP302A1v2	AAEL015655	TTTCGATGTACGGTTGGACA	GCTTTTCGATACGCTGGAGTC
	CYP314A1	AAEL010946	GCGGAGACAAGCAAAAGAAC	ACGATTTCGGCGATTGTATC
	CYP315A1	AAEL011850	ATTCAATTGGACGCTTTTGG	TCCCTTCGTAAACACCTTTG
	P450 reductase	AAEL003349	TTCCCTCCCGCTTTTATCT	CTGTGTAGCGGTGCTTGTGT
	CYP4D24	AAEL007815	CCGCTCGAGCAAAATGCTTATCT	CTAGCTCGAGCCGCAACCTGCT
Drosophila constructs			TATTGGCT	TCTGATCCT
	CYP4H29	AAEL007830	CCGGAATTCCAAATGCTGCCT	CTAGCTCGAGTCTGTCGCAAT
			CTTCTGATG	CTTCACAAA
	CYP4J15v1	AAEL013556	CCGGAATTCCAAATGTTGCTT	CTAGCTCGAGTCTCCTCTCAA
Drosophila constructs			ATTCTAACGC	ACCTAACCTC
	CYP4H33	AAEL013798	CCGGAATTCCAAATGGAATTC	CTAGCTCGAGAATCTTTCCA
			CTAACGAAT	CTAGCTTAAT

^a Nomenclature for the cytochrome P450 genes was taken for the *Ae. aegypti* cytochrome P450 database at: <http://drnelson.uthsc.edu/CytochromeP450.html>.

^b Vectorbase *Ae. aegypti* predicted gene set vs. AeegL1.1. (<http://aegypti.vectorbase.org/>).

aegypti (Strode et al. 2008, Pridgeon et al. 2009, Poupardin et al. 2010, Fonseca-Gonzalez et al. 2011, Bariami et al. 2012, Saavedra-Rodriguez et al. 2012, Strode et al. 2012) and other mosquitoes, including *Anopheles gambiae* Giles (Boonseupsekul et al. 2008; McLaughlin et al. 2008; Müller et al. 2008; Stevenson et al. 2011, 2012), have been extensively studied. Collectively, these studies suggest that cytochrome P450s-mediated detoxification play an important role in the resistance of *Ae. aegypti* to pyrethroid insecticides. Multiple studies have investigated the functional role of insect cytochrome P450s (Joussen et al. 2008; Müller et al. 2008; Zhu et al. 2010; Stevenson et al. 2011, 2012; Yang and Liu 2011; Chandor-Proust et al. 2013), especially on cytochrome P450s in families CYP6 and CYP9. However, information on the functional role of family 4 cytochrome P450s in pyrethroid resistance is scarce.

In Puerto Rico, mosquito control has relied heavily on the usage of pyrethroid insecticide permethrin. However, higher and higher concentrations were needed for successful mosquito control. To understand whether any field strain of *Ae. aegypti* in Puerto Rico has developed resistance to permethrin, a field strain was randomly collected from San Juan, Puerto Rico, in October 2008. The objectives of this study were to: 1) determine whether the Puerto Rico strain of *Ae. aegypti* was resistance to permethrin, 2) determine whether cytochrome P450-mediated detoxification was involved in the resistance in this Puerto Rico strain if the strain was resistant to permethrin, 3) identify up-regulated P450 genes in the Puerto Rico strain of *Ae. aegypti* if the strain was resistant to per-

methrin, and 4) determine whether any of the up-regulated family 4 cytochrome P450 gene could confer resistance in *Drosophila* to permethrin through transgenic work.

Materials and Methods

Mosquito Strains. The Orlando strain of *Ae. aegypti* has been continuously reared at the Center for Medical, Agricultural, and Veterinary Entomology (CMAVE), U.S. Department of Agriculture–Agricultural Research Service (USDA–ARS) in Gainesville, FL, since 1952 (Allan 2011, Clark et al. 2011). The Puerto Rico strain of *Ae. aegypti* was collected in urban San Juan, Puerto Rico in October 2008. Both mosquito strains were reared in the Insectary of the Mosquito and Fly Research Unit at CMAVE, USDA–ARS. Female adults were used for all experiments because only females take bloodmeals and are of concern to the general public. Eggs were hatched by placing a square of a paper towel with eggs in a flask filled with 1,000 ml of distilled water containing 40 mg of larval diet (3:2 brewer's yeast : liver powder [MP Biomedicals, Irvine, CA]). The hatched larvae were held overnight in the flask and 200 larvae were transferred to a 4-liter plastic tray containing 2 liters of distilled water. Larval diet was added to each tray according to the following schedule: Day 1, 80 mg; D 3, 40 mg; D 4, 80 mg; D 5, 120 mg; and D 6, 150 mg. Mosquitoes were reared in an environmental chamber set with a temperature profile representing a simulated summer day regime (ranging from 22 to 30°C) and 80% relative

Table 2. Resistance ratio of the Puerto Rico *Ae. aegypti* strain compared with the Orlando strain in the presence or absence of the P450 inhibitor PBO

Strain	LD ₅₀ (95% CI) μ g per insect	Slope (SE)	χ^2	df	Fold resistance
Orlando					
–PBO	2.08 by 10^{-4} (1.18 by 10^{-4} – 4.11 by 10^{-4})	2.89 (0.44)	8.02	4	1.00
+PBO	1.42 by 10^{-4} (8.80 by 10^{-5} – 2.26 by 10^{-4})	2.08 (0.29)	6.37	5	0.68
Puerto Rico					
–PBO	1.52 by 10^{-2} (9.86 by 10^{-3} – 2.99 by 10^{-2})	1.48 (0.26)	1.39	7	73.07
+PBO	3.28 by 10^{-3} (2.02 by 10^{-3} – 5.85 by 10^{-3})	0.96 (0.14)	3.99	7	15.76

humidity. Incandescent lights were set to a crepuscular profile with a photoperiod of 14:10 (L:D) h, including 2 h of simulated dawn and 2 h of simulated dusk. Adults were held in a screened cage and provided 10% sucrose ad libitum.

Topical Application Bioassays. Topical application bioassays were performed using published procedures (Pridgeon et al. 2007). Briefly, 2- to 5-d-old adults were collected by gentle aspiration, anesthetized at 4°C for 60 min, then females were sorted from males and three 250-cc plastic cups containing 10 adult females each were covered with two layers of tulle mesh and provided with cotton balls saturated with 10% sucrose for feeding. In total, three cups (10 insect per cup) were used for each permethrin dose and all experiments were repeated in triplicate. The LD₅₀ values were determined using six concentrations that resulted in mortality ranging from 10 to 90% along with an acetone control and untreated controls. Before application, females were anesthetized for 30 s with CO₂ and placed on a 4°C chill table (BioQuip Products, Rancho Dominguez, CA). A 0.5- μ l droplet of either acetone (controls), or permethrin dissolved in acetone was applied directly to the dorsal surface of the thorax using a 700 series syringe (Hamilton, Reno, NV). To determine whether cytochrome P450s were involved in the resistance, piperonyl butoxide (PBO, the inhibitor of P450s) was applied topically to adult female *Ae. aegypti* 1 h before the application of the permethrin to allow for the PBO to inhibit the cytochrome P450 activity in the mosquitoes. The dose of 0.4 μ g per mosquito was used for bioassays in the presence of a PBO inhibitor because it was the dose that resulted in no mortality in Orlando or Puerto Rico strains of *Ae. aegypti*.

RNA Extraction, cDNA Synthesis, Primer Design, and Quantitative Polymerase Chain Reaction (qPCR). Total RNA was isolated from Orlando strain for Puerto Rico strain of *Ae. aegypti* using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. First strand cDNA synthesis was conducted on 5 μ g of total RNA in a 20 μ l reaction mixture using oligo-dT₂₀ primer (Invitrogen, Carlsbad, CA). The resulting cDNA was further diluted fivefold as described previously (Pridgeon et al. 2009). qPCR was performed using the SYBR Green PCR Master Mix on an ABI 7300 quantitative PCR System (Applied Biosystems, Foster City, CA). The template used to design primers (Table 1) was based on the P450 sequences of the Liverpool strain of *Ae. aegypti* (GenBank accession no. CH478182). For all cDNA samples, *Ae. aegypti*

actin (GenBank accession no. DQ440059) primers were included as an internal control to normalize the variation of cDNA amount as described previously (Pridgeon et al. 2009). Primers used for the amplification of the actin gene were Actin-152F (5'-AGG-ACCTCGTACGTCGGTGAC-3') and Actin-590R (5'-CGTTCAGTCAGGATCTTC-3'). The qPCR thermal cycling parameters were 50°C for 2 min, 95°C for 10 min, followed by 40 cycle of 95°C for 15 s and 60°C for 1 min. All qPCR was replicated three times. The relative expression level of each of the cytochrome P450 genes was normalized to actin within mosquito strain and then the fold change in gene expression level in Puerto Rico strain compared with that in Orlando strain was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). Statistical analysis was performed using a Welch's *t*-test in R (R Core Team 2013).

Functional Analysis of Four Selected Cytochrome P450 Genes. Four up-regulated family 4 cytochrome P450 genes, *CYP4D24*, *CYP4H29*, *CYP4J15v1*, and *CYP4H33*, were used in this study. The full lengths of the four up-regulated P450 genes from the Puerto Rico strain of *Ae. aegypti* were amplified from cDNA using platinum *Taq*DNA polymerase High Fidelity (Invitrogen, Carlsbad, CA) with gene-specific primers (Table 1) based on the 5' and 3' end sequences of the genes. PCR products of the full length genes were purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The purified PCR products were ligated into pCR 2.1 vector using the Original TA Cloning kit (Invitrogen, Carlsbad, CA) as described by the manufacturer. The full lengths of the genes were cloned in One Shot TOPO 10F' cells using the One Shot TOP10F' Chemically Competent *E. coli* kit (Invitrogen, Carlsbad, CA). Cloning and sequence analyses of the cDNAs were repeated at least three times and three TA clones from each replication were verified by sequencing. The clones were then subcloned into the pUASTattB vector (a gift from Dr. Johannes Bischof, University of Zurich; Brand and Perrimon 1993, Bischof et al. 2007). The plasmid of each pUASTattB-up-regulated gene was transformed into the germ line of *Drosophila melanogaster* Meigen (Bloomington stock #24484, genotype M[vas-int.Dm]ZH-2A, M[3xP3-RFP.attP]ZH-58A), resulting in site-specific integration on chromosome 2R (Bateman et al. 2006; Rainbow Transgenic Flies Inc., Camarillo, CA). Flies were then reciprocally crossed against a W¹¹¹⁸ strain to obtain transgenic *D. melanogaster* with the orange eye phenotype. The flies were then balanced against a *D. melanogaster* balancer

Table 3. Relative cytochrome P450 gene expression values in the pyrethroid-resistant Puerto Rico strain compared with the pyrethroid-susceptible Orlando strain

P450 ^a	AAEL ^b gene no.	Fold up-regulated in Puerto Rico compared with Orlando
CYP15B1	AAEL002067	2.51 ± 0.15 [†]
CYP18A1	AAEL004870	1.29 ± 0.21
CYP303A1ae	AAEL012144	1.15 ± 0.16
CYP304B2xx/yy	AAEL014412	1.29 ± 0.17
CYP304B3yy/xx	AAEL014411	1.00 ± 0.23
CYP304C1	AAEL014413	1.33 ± 0.17
CYP305A6	AAEL002071	1.41 ± 0.26
CYP305A5	AAEL002043	1.38 ± 0.39
CYP306A1	AAEL004888	0.86 ± 0.33
CYP307A1	AAEL009762	1.30 ± 0.28
CYP307A1	AAEL009768	1.48 ± 0.28
CYP307B1	AAEL006875	1.29 ± 0.17
CYP6F2	AAEL014678	4.38 ± 1.38 [†]
CYP6F3	AAEL014684	1.92 ± 0.62
CYP6M5	AAEL009117	0.80 ± 0.38
CYP6M6	AAEL009128	0.69 ± 0.21
CYP6M10	AAEL009125	0.99 ± 0.35
CYP6M11	AAEL009127	5.70 ± 0.44 [†]
CYP6N6	AAEL009126	1.62 ± 0.16
CYP6N9	AAEL009121	2.31 ± 0.17 [†]
CYP6N11	AAEL009119	0.33 ± 0.01 [†]
CYP6N11	AAEL009138	1.50 ± 0.23
CYP6N12	AAEL009124	3.38 ± 0.24 [†]
CYP6N13	AAEL009137	3.53 ± 0.31 [†]
CYP6N14	AAEL009133	1.45 ± 0.12
CYP6N15	AAEL009122	1.61 ± 0.17
CYP6N16	AAEL010151	1.42 ± 0.13
CYP6N17	AAEL010158	1.48 ± 0.25
CYP6P12v1	AAEL012491	1.46 ± 0.13
CYP6S3	AAEL009120	1.7 ± 0.09
CYP6Y3	AAEL009132	1.5 ± 0.10
CYP6Z6	AAEL009123	3.16 ± 0.07 [†]
CYP6Z7	AAEL009130	0.89 ± 0.70
CYP6Z8	AAEL009131	0.06 ± 0.01 [†]
CYP6Z9	AAEL009129	1.31 ± 0.06
CYP6AA5v1	AAEL012492	1.61 ± 0.09
CYP6AC3	AAEL007024	2.33 ± 0.06 [†]
CYP6AC5	AAEL006984	1.18 ± 0.14
CYP6AC6	AAEL006992	1.29 ± 0.16
CYP6AC7	AAEL006989	2.28 ± 0.16 [†]
CYP6AC8	AAEL003890	1.37 ± 0.19
CYP6AH1	AAEL007473	1.51 ± 0.11
CYP6AH1	AAEL015641	1.74 ± 0.26
CYP6AK1	AAEL004941	1.92 ± 0.19
CYP6AL1	AAEL008889	2.06 ± 0.11
CYP6AL3	AAEL009656	0.83 ± 0.16
CYP6BB2	AAEL014893	3.01 ± 0.25 [†]
CYP6BZ1	AAEL012494	1.67 ± 0.15
CYP6CA1	AAEL014680	2.15 ± 0.64
CYP6CB1	AAEL002046	14.46 ± 0.05 [†]
CYP6CB1	AAEL009018	10.60 ± 0.13 [†]
CYP6CB2	AAEL002872	0.52 ± 0.32
CYP6CC1v1	AAEL014890	n/d
CYP6CD1	AAEL005006	1.52 ± 0.23
CYP9J2	AAEL006805	12.17 ± 1.23 [†]
CYP9J6	AAEL002638	1.86 ± 0.18
CYP9J7	AAEL014606	1.72 ± 0.17
CYP9J8	AAEL006811	2.11 ± 0.82
CYP9J9	AAEL006793	2.45 ± 0.39
CYP9J9	AAEL014605	2.83 ± 0.26
CYP9J10	AAEL006798	2.89 ± 0.28 [†]
CYP9J15	AAEL006795	0.70 ± 0.53
CYP9J16	AAEL006815	0.88 ± 0.39
CYP9J17	AAEL009699	0.78 ± 0.24
CYP9J17	AAEL006784	0.31 ± 0.06 [†]
CYP9J18v1	AAEL006804	0.77 ± 0.41
CYP9J19	AAEL006810	1.41 ± 0.20
CYP9J19	AAEL014611	0.04 ± 0.01 [†]
CYP9J20v1	AAEL006814	3.29 ± 0.44 [†]
CYP9J20v2	AAEL014604	2.04 ± 0.50

Table 3. Continued

P450 ^a	AAEL ^b gene no.	Fold up-regulated in Puerto Rico compared with Orlando
CYP9J21	AAEL014612	3.94 ± 0.43 [†]
CYP9J22	AAEL006802	2.30 ± 0.32
CYP9J23	AAEL014615	3.77 ± 0.31 [†]
CYP9J24	AAEL014613	1.84 ± 0.35
CYP9J26	AAEL014609	2.76 ± 0.24 [†]
CYP9J27	AAEL014616	2.85 ± 0.16 [†]
CYP9J27	AAEL014607	3.17 ± 0.19 [†]
CYP9J28	AAEL014617	1.36 ± 0.3
CYP9J29	AAEL014610	0.39 ± 0.07 [†]
CYP9J30	AAEL014603	1.37 ± 0.33
CYP9J31	AAEL002633	2.30 ± 0.14 [†]
CYP9J32	AAEL008846	1.16 ± 0.15
CYP9M4	AAEL001320	1.02 ± 0.30
CYP9M5	AAEL001288	1.18 ± 0.34
CYP9M6	AAEL001312	1.83 ± 0.23
CYP9M7	AAEL001292	1.73 ± 0.38
CYP9M8	AAEL009591	1.55 ± 0.21
CYP9M9	AAEL001807	1.01 ± 0.14
CYP9AE1	AAEL003748	0.38 ± 0.08 [†]
CYP329B1	AAEL003763	1.51 ± 0.13
CYP4C38	AAEL012266	1.09 ± 0.15
CYP4C50	AAEL008017	1.20 ± 0.19
CYP4C51	AAEL008018	0.85 ± 0.36
CYP4C52	AAEL008023	0.61 ± 0.42
CYP4D23	AAEL007816	1.68 ± 0.17
CYP4D24	AAEL007815	2.81 ± 0.20 [†]
CYP4D37	AAEL007795	1.47 ± 0.30
CYP4D38	AAEL007807	1.44 ± 0.10
CYP4D39	AAEL007808	1.54 ± 0.07
CYP4G35	AAEL008345	3.03 ± 0.07 [†]
CYP4G35	AAEL006824	3.44 ± 0.20 [†]
CYP4G36	AAEL004054	2.84 ± 0.16 [†]
CYP4H28	AAEL003380	1.27 ± 0.71
CYP4H29	AAEL007830	2.52 ± 0.43 [†]
CYP4H30	AAEL003399	3.85 ± 0.50 [†]
CYP4H31	AAEL002085	1.36 ± 0.51
CYP4H32	AAEL007812	0.36 ± 0.02 [†]
CYP4H33	AAEL013798	2.73 ± 0.13 [†]
CYP4J13	AAEL013555	1.05 ± 0.15
CYP4J14	AAEL013554	1.00 ± 0.11
CYP4J15v1	AAEL013556	2.30 ± 0.18 [†]
CYP4J15v2	AAEL014829	3.85 ± 0.46 [†]
CYP4J16	AAEL015663	1.06 ± 0.29
CYP4J17	AAEL015370	1.23 ± 0.42
CYP4J17	AAEL014019	0.44 ± 0.03 [†]
CYP4K3	AAEL007798	1.19 ± 0.28
CYP4AR2	AAEL010154	1.09 ± 0.23
CYP325E3	AAEL000338	0.76 ± 0.64
CYP325G2	AAEL012766	2.06 ± 0.31
CYP325G3	AAEL012772	3.61 ± 1.20 [†]
CYP325K2	AAEL005771	1.23 ± 0.24
CYP325K3	AAEL005788	2.08 ± 0.19
CYP325L1v1	AAEL011770	1.66 ± 0.07
CYP325M1	AAEL012773	1.28 ± 0.18
CYP325M2	AAEL012769	1.41 ± 0.19
CYP325M2	AAEL015591	1.54 ± 0.19
CYP325M3	AAEL012765	0.31 ± 0.02 [†]
CYP325M4	AAEL011769	2.72 ± 0.28 [†]
CYP325M5	AAEL011761	1.40 ± 0.18
CYP325N1	AAEL012770	0.76 ± 0.34
CYP325N2	AAEL012762	0.98 ± 0.14
CYP325P1	AAEL000340	1.66 ± 0.13
CYP325Q1	AAEL006044	1.16 ± 0.13
CYP325Q2	AAEL015563	1.44 ± 0.30
CYP325R1	AAEL005775	1.76 ± 0.09
CYP325S1	AAEL000326	0.51 ± 0.41
CYP325S2	AAEL000325	0.68 ± 0.10
CYP325S3	AAEL000357	0.56 ± 0.05
CYP325T2	AAEL012761	1.02 ± 0.22
CYP325T2	AAEL015475	0.81 ± 0.13

Continued on following page

Table 3. Continued

P450 ^a	AAEL ^b gene no.	Fold up-regulated in Puerto Rico compared with Orlando
CYP325U1	AAEL000320	0.14 ± 0.01
CYP325X1	AAEL005695	1.19 ± 0.13
CYP325X2	AAEL005696	1.37 ± 0.12
CYP325X4	AAEL005700	1.39 ± 0.14
CYP325Y1	AAEL006257	1.09 ± 0.20
CYP325Y2v2	AAEL015362	0.50 ± 0.23
CYP325Y3	AAEL006246	1.21 ± 0.20
CYP325Y3	AAEL015361	1.31 ± 0.22
CYP325Z1	AAEL010273	1.30 ± 0.13
CYP325AA1	AAEL004012	1.51 ± 0.21
CYP12F5	AAEL001960	0.47 ± 0.27
CYP12F6	AAEL002005	2.14 ± 0.19*
CYP12F7	AAEL002031	1.37 ± 0.11
CYP12F8	AAEL006827	2.30 ± 0.29†
CYP49A1	AAEL008638	1.45 ± 0.14
CYP301A1	AAEL014594	1.84 ± 0.15
CYP302A1v1	AAEL011463	1.91 ± 0.15
CYP302A1v2	AAEL015655	1.46 ± 0.22
CYP314A1	AAEL010946	0.77 ± 0.16
CYP315A1	AAEL011850	1.92 ± 0.07
NADPH P450 reductase	AAEL003349	4.76 ± 0.18†

^a Nomenclature for the cytochrome P450 genes was taken for the *Ae. aegypti* cytochrome P450 database at: <http://dmelson.uthsc.edu/CytochromeP450.html>.

^b Vectorbase *Ae. aegypti* predicted gene set vs. AaegL1.1. <http://aaegypti.vectorbase.org/>.

* Significantly up-regulated (more than twofold) at the $P < 0.05$ level of significance.

† Significantly up-regulated (more than twofold) at the $P < 0.01$ level of significance.

‡ Significantly down-regulated (more than twofold) at the $P < 0.01$ level of significance.

strain (Bloomington stock #6312, genotype: w[1118]/Dp(1;Y)y[+]; sna[Sco]/CyO, P[ry[+t7.2] = sevRas1.V12]FK1) to generate a homozygous line containing the cytochrome P450 transgene on chromosome 2R. The insertion of the up-regulated genes in the transgenic fruit fly lines were further confirmed using reverse transcription-polymerase chain reaction (RT-PCR). Transgenic virgin female *D. melanogaster* were then crossed with male GAL4-expressing *D. melanogaster* (Bloomington stock #3954, genotype: P[Act5C-GAL4]17bFO1), which expresses GAL4 under control of the Act5C promoter, resulting in ubiquitous nontissue-specific expression. The F1 generation of these crosses expressed GAL4 and contained a single copy of the cytochrome P450 transgene, which was under control of the upstream activating sequence (UAS) enhancer. Permethrin toxicity bioassays were then conducted on 2–3-d posteclosion female *Drosophila* of F1 UAS-GAL4 crosses to examine the toxicity of pyrethroids to transgenic flies. Briefly, serial concentrations of each pyrethroid solution in acetone, ranging from 25 to 100 ng/μl that gave >0 and <100% mortality to the tested insects were prepared. Two hundred microliter of each permethrin concentration solution was evenly coated on the inside of individual 20-ml glass scintillation vials. Then, 15 female flies were transferred to each of the prepared vials, and three vials were used for each concentration for each bioassay replicate. The vials were plugged with cotton balls soaked with 5% sucrose and the mortality was scored after a 24-h expo-

sure to the pyrethroids. Each bioassay was independently replicated three times using only flies that exhibited the correct morphological marker (GAL4 red eyes). The *D. melanogaster* strain (Bloomington stock #24484, genotype: M[vas-int.Dm]ZH-2A, M[3xP3-RFP.attP]ZH-58A) containing the empty pUAST vector donated insert, but no transgene was used as the control reference strain according to the identical crossing protocol of virgin control females with GAL4 expressing males to obtain the F1 generation for testing. Preliminary testing determined that vials coated with 2 μg of permethrin resulted in nearly complete mortality of the empty-vector control line. Subsequently, the lowest insecticide concentration at 5 μg of permethrin resulted in 100% mortality of the control mosquitoes for all bioassay replicates. Therefore, the concentration of 5 and 10 μg per vial of permethrin were used to test the transgenic flies' susceptibility to permethrin. All tests were run at 27°C and mortality was assessed at 24 h postexposure. All *D. melanogaster* were reared on Jazz-Mix *Drosophila* food (Fisher, KS City, MO) at 25 ± 2°C under a photoperiod of 12:12 (L:D) h following standard protocols (Ashburner et al. 2005).

Results and Discussion

Results of the topical application bioassays are summarized in Table 2. The LD₅₀ value of permethrin to Orlando strain of *Ae. aegypti* was 2.08 by 10⁻⁴ μg per mosquito, whereas that of permethrin to the Puerto Rico strain of *Ae. aegypti* was 1.52 by 10⁻² μg per mosquito (Table 2). Therefore, the Puerto Rico strain of *Ae. aegypti* was 73-fold resistant to permethrin compared with that of the Orlando strain (Table 2). When PBO (the inhibitor of cytochrome P450) was present, the resistance of the Puerto Rico strain to permethrin was decreased to 15-fold (Table 2). These results suggested that cytochrome P450-mediated detoxification might play a role in the resistance of the Puerto Rico strain of *Ae. aegypti* to permethrin.

Results of transcriptional levels of P450 genes determined by qPCR were summarized in Table 3. Of the 164 cytochrome P450s selected for this study, 33 were significantly ($P < 0.05$) up-regulated more than twofold (Table 3) and eight were significantly ($P < 0.05$) down-regulated more than twofold (Table 3). For the remaining 123 cytochrome P450 genes, no significant difference ($P > 0.05$) was observed between the transcriptional level in the Puerto Rico strain and that of the Orlando strain (Table 3). Although the Puerto Rico field population is geographically distinct from the Orlando strain, the transcriptional levels of the majority (123 of 164) of the P450s in the two strains were not significantly different, suggesting that not all P450s play roles in resistance to permethrin. Our results also further suggest that resistance is developed under selection pressure, as permethrin was widely used in Puerto Rico to control mosquitoes.

Previous studies by multiple researchers have discovered the up-regulation of cytochrome P450 genes in insecticide-resistant *Ae. aegypti* (Strode et al. 2008, Marcombe et al. 2009, Bariami et al. 2012, Saavedra-Rodri-

Table 4. Transcriptional level of differentially expressed cytochrome in the Puerto Rico strain compared with that in the Orlando strain of *Ae. aegypti*

Gene ^b	Name ^a	Fold	Reported up-regulation in insecticide-resistant <i>Ae. aegypti</i>
AAEL002067	<i>CYP15B1</i>	2.51 ± 0.03 [†]	
AAEL007024	<i>CYP6AG3</i>	2.33 ± 0.05 [†]	
AAEL006989	<i>CYP6AG7</i>	2.28 ± 0.08 [†]	
AAEL014893	<i>CYP6BB2</i>	3.01 ± 0.18 [†]	(Bariami et al. 2012, Saavedra-Rodriguez et al. 2012)
AAEL002046	<i>CYP6CB1</i>	14.46 ± 0.28 [†]	(Strode et al. 2008, Bariami et al. 2012, Saavedra-Rodriguez et al. 2012)
AAEL009018	<i>CYP6CB1</i>	10.60 ± 0.41 [†]	(Strode et al. 2008, Bariami et al. 2012)
AAEL014678	<i>CYP6F2</i>	4.38 ± 0.11 [†]	
AAEL009127	<i>CYP6M11</i>	5.70 ± 0.31 [†]	(Poupardin et al. 2008, Marcombe et al. 2009, Bariami et al. 2012)
AAEL009121	<i>CYP6N9</i>	2.31 ± 0.04 [†]	(Bariami et al. 2012)
AAEL009124	<i>CYP6N12</i>	3.38 ± 0.16 [†]	(Bariami et al. 2012)
AAEL009137	<i>CYP6N13</i>	3.53 ± 0.01 [†]	
AAEL009123	<i>CYP6Z6</i>	3.16 ± 0.02 [†]	(Marcombe et al. 2009, Saavedra-Rodriguez et al. 2012)
AAEL006805	<i>CYP9J2</i>	12.17 ± 2.83 [†]	
AAEL006798	<i>CYP9J10</i>	2.89 ± 0.29 [†]	(Strode et al. 2008, Bariami et al. 2012)
AAEL006814	<i>CYP9J20v1</i>	3.29 ± 0.20 [†]	
AAEL014612	<i>CYP9J21</i>	3.94 ± 0.42 [†]	
AAEL014609	<i>CYP9J26</i>	2.76 ± 0.22 [†]	(Strode et al. 2008, Bariami et al. 2012)
AAEL014616	<i>CYP9J27</i>	2.85 ± 0.13 [†]	(Strode et al. 2008, Bariami et al. 2012)
AAEL002633	<i>CYP9J31</i>	2.30 ± 0.07 [†]	
AAEL013556	<i>CYP4J15v1</i>	2.30 ± 0.06 [†]	(Marcombe et al. 2009 [†])
AAEL014829	<i>CYP4J15v2</i>	3.85 ± 0.22 [†]	(Marcombe et al. 2009 [†])
AAEL007815	<i>CYP4D24</i>	2.81 ± 0.09 [†]	
AAEL008345	<i>CYP4G35</i>	3.03 ± 0.08 [†]	
AAEL006824	<i>CYP4G35</i>	3.44 ± 0.30 [†]	
AAEL007830	<i>CYP4H29</i>	2.52 ± 0.18 [†]	
AAEL003399	<i>CYP4H30</i>	3.85 ± 0.18 [†]	
AAEL013798	<i>CYP4H33</i>	2.73 ± 0.03 [†]	
AAEL004054	<i>CYP4G36</i>	2.84 ± 0.23 [†]	(Saavedra-Rodriguez et al. 2012)
AAEL012766	<i>CYP325G2</i>	2.06 ± 0.04	
AAEL012772	<i>CYP325G3</i>	3.61 ± 0.20 [†]	
AAEL011769	<i>CYP325M4</i>	2.72 ± 0.11 [†]	
AAEL002005	<i>CYP12F6</i>	2.14 ± 0.06*	(Strode et al. 2008)
AAEL006827	<i>CYP12F8</i>	2.30 ± 0.06 [†]	
AAEL009119	<i>CYP6N11</i>	0.33 ± 0.01 [†]	
AAEL009131	<i>CYP6Z8</i>	0.06 ± 0.01 [†]	
AAEL006784	<i>CYP9J17</i>	0.31 ± 0.06 [†]	
AAEL014611	<i>CYP9J19</i>	0.04 ± 0.01 [†]	
AAEL014610	<i>CYP9J29</i>	0.39 ± 0.07 [†]	
AAEL003748	<i>CYP9AE1</i>	0.38 ± 0.08 [†]	
AAEL007812	<i>CYP4H32</i>	0.36 ± 0.02 [†]	
AAEL014019	<i>CYP4J17</i>	0.44 ± 0.03 [†]	
AAEL012765	<i>CYP325M3</i>	0.31 ± 0.02 [†]	

^a Nomenclature for the cytochrome P450 genes was taken for the *Ae. aegypti* cytochrome P450 database at: <http://drnelson.uthsc.edu/CytochromeP450.html>.

^b Vectorbase *Ae. aegypti* predicted gene set v. AaegL1.1. <http://aaegypti.vectorbase.org/>.

* Significantly up-regulated (more than twofold) at the $P < 0.05$ level of significance.

[†] Significantly up-regulated (more than twofold) at the $P < 0.01$ level of significance.

[†] Significantly down-regulated (more than twofold) at the $P < 0.01$ level of significance.

guez et al. 2012). Of the 33 up-regulated P450 genes, the following 13 were also reported in literature: *CYP6BB2*, *CYP6CB1*, *CYP6M1*, *CYP6N9*, *CYP6N12*, *CYP6Z6*, *CYP9J10*, *CYP9J26*, *CYP9J27*, *CYP4J15v1*, *CYP4J15v2*, *CYP4G36*, and *CYP12F6* (Table 4). Multiple studies have investigated the functional role of insect cytochrome P450s (Jousen et al. 2008; Müller et al. 2008, 2011; Zhu et al. 2010; Stevenson et al. 2011, 2012; Yang and Liu, 2011; Chandor-Proust et al. 2013), especially on cytochrome P450s in families CYP6 and CYP9. For example, Stevenson et al. (2012) investigated the function of seven family six and nine cytochrome P450s in *Ae. aegypti*, while multiple other studies have investigated family six and nine in other mosquito species as well (Boonsuepsakul et al. 2008, Duangkaew et al. 2011, Lertkitatmongkol et al. 2011). However, information on the functional role of cytochrome P450s family four in

pyrethroid resistance is scarce. Therefore, four genes (*CYP4D24*, *CYP4H29*, *CYP4J15v1*, and *CYP4H33*) from family CYP4 were selected for further functional studies, of which only *CYP4J15v1* has been previously reported to be up-regulated in permethrin-resistant *Ae. aegypti* (Marcombe et al. 2009), whereas the other three family four P450s have not been previously linked to insecticide resistance in *Ae. aegypti*. After successful full length cloning (Fig. 1) and sequence confirming, the four P450 genes from the Puerto Rico strain of *Ae. aegypti* were transferred and expressed in *D. melanogaster* under control of the GAL4-UAS enhancer trap system. When adult female *D. melanogaster* were exposed to permethrin at a concentration of 5 µg per vial, none of the control *D. melanogaster* (empty vector nontransgenic control) survived. However, when exposed to permethrin at 5 µg per

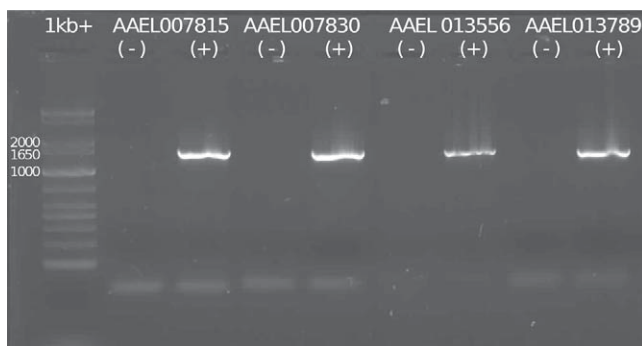


Fig. 1. RT-PCR of transgenic *D. melanogaster* expressing *Ae. aegypti* cytochrome P450 genes. The (-) and (+) within gene represent the amplified products from the nontransgenic empty vector control line (-) and the transgenic line (+) of *D. melanogaster*, respectively.

vial, transgenic *D. melanogaster* expressing *CYP4D24*, *CYP4H29*, *CYP4J15v1*, and *CYP4H33* had a survival rate of 60.0 ± 6.7 , 29.0 ± 4.4 , 64.4 ± 9.7 , and $11.0 \pm 4.4\%$, respectively (Fig. 2). When exposed to permethrin at a higher concentration (10 μg per vial), none of the control *D. melanogaster* survived. Similarly, none of the transgenic *D. melanogaster* expressing *CYP4J15v1* or *CYP4H33* survived when they were exposed to permethrin at 10 μg per vial. However, transgenic *D. melanogaster* expressing *CYP4D24* and *CYP4H29* had a survival rate of 37.8 ± 4.4 and $2.2 \pm 2.2\%$, respectively (Fig. 2). Taken together, these results suggest that all these four P450s play some roles in the resistance of the Puerto Rico strain to permethrin, with *CYP4D24* playing a bigger role than the other three family 4 P450s used in this study. However, the fact that transgenic *D. melanogaster* expressing *CYP4D24*, *CYP4H29*, *CYP4J15v1*, and *CYP4H33* had a significantly higher survival rate when exposed to permethrin at 5 μg per vial compared with that when exposed to permethrin

at 10 μg per vial, suggesting that a single P450 gene might only play a partial role in the resistance to permethrin.

In conclusion, topical application bioassay revealed that the Puerto Rico strain of *Ae. aegypti* was 73-fold resistant to permethrin compared with the Orlando strain. In the presence of the cytochrome P450's inhibitor PBO, the resistance of Puerto Rico strain of *Ae. aegypti* was reduced to 15-fold, suggesting that cytochrome P450-mediated detoxification mechanism is involved in the resistance of the Puerto Rico strain of *Ae. aegypti* to permethrin. Of the 164 selected cytochrome P450s, 33 were significantly up-regulated more than twofold. Functional studies using *D. melanogaster* as a model insect, four family 4 cytochrome P450s selected for this study were found to confer some resistance to permethrin. When exposed to 5 μg per vial permethrin, transgenic *D. melanogaster* expressing *CYP4D24*, *CYP4H29*, *CYP4J15v1*, and *CYP4H33* had a survival rate of 60.0 ± 6.7 , 29.0 ± 4.4 , 64.4 ± 9.7 , and $11.0 \pm 4.4\%$, respectively. However, none of the control flies survived the permethrin exposure at the same concentration. Similarly, none of the transgenic *D. melanogaster* expressing *CYP4J15v1* or *4H33* survived when they were exposed to permethrin at 10 μg per vial. However, transgenic *D. melanogaster* expressing *CYP4D24* and *CYP4H29* had a survival rate of 37.8 ± 4.4 and $2.2 \pm 2.2\%$, respectively. Taken together, our results suggest that *CYP4D24* might play an important role in cytochrome P450-mediated resistance to permethrin.

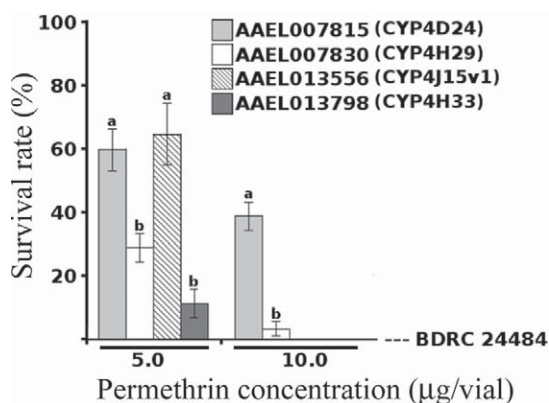


Fig. 2. Survival rate of transgenic *D. melanogaster* lines following a 24 h exposure to permethrin. Bars within dose superseded by the same letter are not significantly different ($P > 0.05$) whereas bars with different letter are significantly different ($P < 0.05$). BDRC 2,4484 is the nontransgenic empty vector control line of *D. melanogaster*, which had no survival after exposure to the two concentrations of permethrin used in this study.

Acknowledgments

We thank Neil Sanscrainte, Matthew Brown, Francis Golden, and Nathan Newlon for their assistance with the rearing of the mosquitoes. We also thank the Deployed War-Fighter Protection program for funding.

References Cited

- Allan, S. 2011. Susceptibility of adult mosquitoes to insecticides in aqueous sucrose baits. *J. Vector Ecol.* 36: 59–67.
- Ashburner, M., K. G. Golic, and R. S. Hawley. 2005. *Drosophila: a laboratory handbook*. Cold Spring Harbor Laboratory Press, Woodbury, NY.

- Bariami, V., C. M. Jones, R. Poupardin, J. Vontas, and H. Ranson. 2012. Gene amplification, ABC transporters and Cytochrome P450s: unraveling the molecular basis of Pyrethroid resistance in the dengue vector, *Aedes aegypti*. *PLoS Negl. Trop. Dis.* 6: e1692. (doi:10.1371/journal.pntd.0001692).
- Bateman, J. R., A. M. Lee, and C. Wu. 2006. Site-specific transformation of *Drosophila* via phiC31 integrase-mediated cassette exchange. *Genetics* 173:769–77. (doi:10.1534/genetics.106.056945).
- Bischof, J., R. K. Maeda, M. Hediger, F. Karch, and K. Basler. 2007. An optimized transgenesis system for *Drosophila* using germ-line-specific phiC31 integrases. *Proc. Natl. Acad. Sci. U.S.A.* 104: 3312–3317. (doi:10.1073/pnas.0611511104).
- Boonsuepsakul, S., E. Luepromchai, and P. Rongnparut. 2008. Characterization of *Anopheles minimus* CYP6AA3 expressed in a recombinant baculovirus system. *Arch. Insect Biochem. Physiol.* 69: 13–21. (doi:10.1002/arch.20248).
- Brand, A. H., and N. Perrimon. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118: 401–415.
- Chandor-Proust, A., J. Bibby, M. Régent-Kloeckner, J. Roux, E. Guittard-Crilat, R. Poupardin, M. A. Riaz, M. Paine, C. Dauphin-Villemant, S. Reynaud, et al. 2013. The central role of mosquito in insecticide detoxification revealed by functional expression and structural modelling. *Biochem. J. cytochrome P450 CYP6Zs* 455: 75–85.
- Clark, G. G., U. R. Bernier, S. A. Allan, D. L. Kline, and F. V. Golden. 2011. Changes in host-seeking behavior of Puerto Rican *Aedes aegypti* after colonization. *J. Med. Entomol.* 48: 533–537. (doi:10.1603/MEI0207).
- Curtis, C. F., J. E. Miller, M. H. Hodjati, J. H. Kolaczinski, and I. Kasumba. 1998. Can anything be done to maintain the effectiveness of pyrethroid-impregnated bednets against malaria vectors? *Phil. Trans. R. Soc. London B* 353: 1769–1775.
- Feyereisen, R. 1995. Molecular biology of insecticide resistance. *Toxicol. Lett.* 82/83: 83–90.
- Ffrench-Constant, R. H., P. J. Daborn, and G. Le Goff. 2004. The genetics and genomics of insecticide resistance. *Trends Genet.* 20: 163–170.
- Fonseca-Gonzalez, I., M. L. Quinones, A. Lenhart, and W. G. Brogdon. 2011. Insecticide resistance status of *Aedes aegypti* (L.) from Colombia. *Pest Manag. Sci.* 67: 430–437.
- Gong, Y., T. Li, L. Zhang, X. Gao, and N. Liu. 2013. Permethrin induction of multiple cytochrome P450 genes in insecticide resistant mosquitoes, *Culex quinquefasciatus*. *Int. J. Biol. Sci.* 9: 863–871.
- Gubler, D. J. 1988. Dengue, pp. 223–260. In T. Monarth (ed.), *The Arboviruses*, vol. 2. Epidemiology and Ecology. CRC, Boca Raton, FL.
- Gubler, D. J., and G. G. Clark. 1995. Dengue/dengue hemorrhagic fever: the emergence of a global health problem. *Emerg. Infect. Dis.* 1: 55–57.
- Hemingway, J., N. J. Hawkes, L. McCarroll, and H. Ranson. 2004. The molecular basis of insecticide resistance in mosquitoes. *Insect Biochem. Mol. Biol.* 34: 653–665.
- Hougard, J. M., S. Duchon, M. Zaim, and P. Guillet. 2002. Bifenthrin: a useful pyrethroid insecticide for treatment of mosquito nets. *J. Med. Entomol.* 39: 526–533.
- Joussen, N., D. G. Heckel, M. Haas, I. Schuphan, and B. Schmidt. 2008. Metabolism of imidacloprid and DDT by P450 CYP6G1 expressed in cell cultures of *Nicotiana tabacum* suggests detoxification of these insecticides in Cyp6g1 overexpressing strains of *Drosophila melanogaster*, leading to resistance. *Pest Manag. Sci.* 64: 65–73.
- Juntarajumnong, W., S. Pimnon, M. J. Bangs, K. Thanispong, and T. Chareonviriyaphap. 2012. Discriminating lethal concentrations and efficacy of six pyrethroids for control of *Aedes aegypti* in Thailand. *J. Am. Mosq. Control Assoc.* 28: 30–37.
- Lertkiatmongkol, P., E. Jenwitheesuk, and P. Rongnparut. 2011. Homology modeling of mosquito cytochrome P450 enzymes involved in pyrethroid metabolism: insights into differences in substrate selectivity. *BMC Res. Notes* 4: 321.
- Liu, N. 2008. Insecticide resistance in mosquitoes: development and mechanisms, pp. 75–91. In N. Liu (ed.), *Recent Advances in Insect Physiology, Toxicology and Molecular Biology*. Research Signpost, Kerala, India.
- Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. *Methods* 25: 402–408.
- Manda H, P. Shah, S. Polsomboon, T. Chareonviriyaphap, F. Castro-Llanos, A. Morrison, R. G. Burrus, J. P. Greico, and N. L. Achee. 2013. Contact irritant responses of *Aedes aegypti* using sublethal concentration and focal application of pyrethroid chemicals. *PLoS Negl. Trop. Dis.* 7: e2074. (doi:10.1371/journal.pntd.0002074).
- Marcombe, S., R. Poupardin, F. Darriet, S. Reynaud, J. Bonnet, C. Strode, C. Brengues, A. Yébakima, H. Ranson, V. Corbel, et al. 2009. Exploring the molecular basis of insecticide resistance in the dengue vector *Aedes aegypti*: a case study in Martinique Island (French West Indies). *BMC Genomics* 10: 494. (doi:10.1186/1471-2164-10-494).
- McLaughlin, L. A., U. Niazi, J. Bibby, J.-P. David, J. Vontas, J. Hemingway, H. Ranson, M. J. Sutcliffe, and M. J. I. Paine. 2008. Characterization of inhibitors and substrates of *Anopheles gambiae* CYP6Z2. *Insect Mol. Biol.* 17: 125–135.
- Müller, P., E. Warr, B. J. Stevenson, P. M. Pignatelli, J. C. Morgan, A. Steven, A. E. Yawson, S. N. Mitchell, H. Ranson, J. Hemingway, et al. 2008. Field-caught permethrin-resistant *Anopheles gambiae* overexpress CYP6P3, a P450 that metabolises pyrethroids. *PLoS Genet.* 4:e1000286.
- Poupardin, R., M. Riaz, J. Vontas, J. P. David, and S. Reynaud. 2010. Transcription profiling of eleven cytochrome P450s potentially involved in xenobiotic metabolism in the mosquito *Aedes aegypti*. *Insect Mol. Biol.* 19: 185–193. (doi:10.1111/j.1365-2583.2009.00967).
- Pridgeon, J. W., K. M. Meepagala, J. J. Becnel, G. G. Clark, R. M. Pereira, and K. J. Linthicum. 2007. Structure-activity relationships of 33 piperidines as toxicants against female adults of *Aedes aegypti* (Diptera: Culicidae). *J. Med. Entomol.* 44: 263–269.
- Pridgeon, J. W., J. J. Becnel, G. G. Clark, and K. J. Linthicum. 2009. Permethrin induces overexpression of multiple genes in *Aedes aegypti*. *J. Med. Entomol.* 46: 580–587.
- R Core Team. 2013. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. (<http://www.R-project.org/>).
- Reid, W. R., L. Zhang, F. Liu, and N. Liu. 2012. The transcriptome profile of the mosquito *Culex quinquefasciatus* following Permethrin selection. *PLoS ONE* 7: e47163. (doi:10.1371/journal.pone.0047163).
- Saavedra-Rodriguez, K., A. F. Suarez, I. F. Salas, C. Strode, H. Ranson, J. Hemingway, and W. C. Black. 2012. Transcription of detoxification genes after permethrin selection in the mosquito *Aedes aegypti*. *Insect Mol. Biol.* 21: 61–77.
- Stevenson, B. J., J. Bibby, P. Pignatelli, S. Muangnoicharoen, P. M. O'Neill, L. Y. Lian, P. Muller, D. Nikou, A. Steven, J. Hemingway, M. J. Sutcliffe, and M. J. I. Paine. 2011. Cytochrome P450 6M2 from the malaria vector *Anopheles gam-*

- biae* metabolizes pyrethroids: sequential metabolism of deltamethrin revealed. *Insect Biochem. Mol. Biol.* 41: 492–502.
- Stevenson, B. J., P. Pignatelli, D. Nikou, and M. J. Paine. 2012. Pinpointing P450s associated with pyrethroid metabolism in the dengue vector, *Aedes aegypti*: developing new tools to combat insecticide resistance. *PLoS Negl. Trop. Dis.* 6: e1595.
- Strode, C., C. S. Wondji, J.-P. David, N. J. Hawkes, N. Lumjuan, D. R. Nelson, D. R. Drane, S.H.P.P. Karunaratne, J. Hemingway, W. C. Black IV, et al. 2008. Genomic analysis of detoxification genes in the mosquito *Aedes aegypti*. *Insect Biochem. Mol. Biol.* 38: 113–123.
- Strode, C., M. de Melo-Santos, T. Magalhães, A. Araújo, and C. Ayres. 2012. Expression profile of genes during resistance reversal in a temephos selected strain of the dengue vector, *Aedes aegypti*. *PLoS ONE* 7: e39439. (doi:10.1371/journal.pone.0039439).
- Vulule, J. M., R. F. Beach, F. K. Atieli, J. M. Roberts, D. L. Mount, and R. W. Mwangi. 1994. Reduced susceptibility of *Anopheles gambiae* to permethrin associated with the use of permethrin-impregnated bednets and curtains in Kenya. *Med. Vet. Entomol.* 8: 71–75.
- Warren, K. S., and A.A.F. Mahmoud. 1990. Tropical and geographical medicine, 2nd ed. McGraw-Hill, New York, NY.
- Yang, T., and N. Liu. 2011. Genome analysis of cytochrome P450s and their expression profiles in insecticide resistant mosquitoes, *Culex quinquefasciatus*. *PLoS ONE* 6: e29418. (doi:10.1371/journal.pone.0029418).
- Zhu, F., R. Parthasarathy, H. Bai, K. Woithe, M. Kaussmann, R. Nauen, D. A. Harrison, and S. R. Palli. 2010. A brain-specific cytochrome P450 responsible for the majority of deltamethrin resistance in the QTC279 strain of *Tribolium castaneum*. *Proc. Natl. Acad. Sci. U.S.A.* 107: 8557–8562.

Received 25 November 2013; accepted 1 February 2014.