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Vector Competence of New Zealand Mosquitoes for Selected Arboviruses

Laura D. Kramer,* Pam Chin, Rachel P. Cane, Elizabeth B. Kauffman, and Graham Mackereth

Wadsworth Center, New York State Department of Health, Albany, New York; School of Public Health, State University of New York, Albany, New York; Mosquito Consulting Services (NZ) Lincoln, New Zealand; ESR NCBID-Wallaceville, Upper Hutt, New Zealand

Abstract. New Zealand (NZ) historically has been free of arboviral activity with the exception of Whataroa virus (*Togaviridae*: *Alphavirus*), which is established in bird populations and is transmitted by local mosquitoes. This naive situation is threatened by global warming, invasive mosquitoes, and tourism. To determine the threat of selected medically important arboviruses to NZ, vector competence assays were conducted using field collected endemic and introduced mosquito species. Four alphaviruses (*Togaviridae*): Barmah Forest virus, Chikungunya virus, Ross River virus, and Sindbis virus, and five flaviviruses (*Flaviviridae*): Dengue virus 2, Japanese encephalitis virus, Murray Valley encephalitis virus, West Nile virus, and Yellow fever virus were evaluated. Results indicate some NZ mosquito species are highly competent vectors of selected arboviruses, particularly alphaviruses, and may pose a threat were one of these arboviruses introduced at a time when the vector was prevalent and the climatic conditions favorable for virus transmission.

INTRODUCTION

New Zealand (NZ) historically has been free of arboviral activity with the exception of Whataroa virus (WHAV) (*Togaviridae*: *Alphavirus*), which is established in bird populations on the west coast of the South Island of NZ and has been isolated from the local mosquitoes, *Culiseta tonnoiri* (Edwards) and *Culex pervigilans* Bergroth in South Westland.[1–3] This naive situation is threatened by global warming, invasive mosquito species, and an increasing level of tourism where travelers and mosquitoes on the aircrafts in which they fly may inadvertently introduce mosquito-borne and other viruses actively circulating in other parts of the world.[4] The speed of travel and the increasing numbers of people traveling create an environment where viruses can readily be introduced into new environments. As a step in determining the potential for endemic mosquitoes currently existing in significant numbers in NZ, and for known invasive species, to serve as vectors of viruses, evaluation of vector competence for selected species and medically significant viruses under appropriate conditions was conducted.

New Zealand has both endemic and introduced mosquito species active on both of the main islands, the North and South Islands.[5] *Opifex fuscus* is one of three endemic species and is the sole member of the *Opifex* genus. This is a coastal mosquito that is found on both the North and South Islands where it lives and oviposits in coastal rock pools.[6] *Culex pervigilans*, a second endemic species, can be found throughout the islands of NZ. Both *Op. fuscus* and *Cx. pervigilans* are seasonally active in the summer. However, another endemic species, the flood water ground pool ovipositing *Aedes antipodeus*, is active during winter months on both the North and South Islands.[7]

Three introduced species found in NZ are *Aedes australis*, *Aedes notoscriptus*, and *Culex quinquefasciatus*, although *Cx. quinquefasciatus* has been present in NZ for ~150 years and may be considered an established exotic species at this stage.[8] *Aedes australis* was introduced to NZ in the 1960s aboard shipping vessels.[9] It has become established in coastal saline rock pools of the southeast South Island and Stewart Island, apparently displacing *Op. fuscus* in these areas (Cane RP, unpublished data), and has remained restricted to these areas to date.[4,8] This cold tolerant species lays eggs from October to April. *Aedes notoscriptus* was first reported in the 1920s and is endemic to Australia.[10] A container ovipositing fresh water species predominantly occurring in urban and semi-urban areas, it can be found throughout the North Island and in isolated areas of the South Island as far south as Christchurch (Cane RP, unpublished data). In temperate climates *Ae. notoscriptus* are active all year, but in the cooler climate of NZ they overwinter as larvae. *Culex quinquefasciatus* was first recorded in NZ in 1848 and it is believed to have been introduced from whaling vessels.[8,11] This species can be found on both the North and the South Islands and resides mostly in and around urban settings. This species is active in the warmer months, overwintering as larvae from May to September.

Only one vector competence study for arboviruses has been conducted with mosquito populations from NZ,[12] but more extensive studies with strains of selected species collected in Australia have been published. These studies examined vector competence of Australian mosquito species for alphaviruses (*Togaviridae*) Barmah Forest virus (BFV), Chikungunya virus (CHIKV), Murray Valley encephalitis virus (MVEV), Ross River virus (RRV), and Sindbis virus (SINV); and flaviviruses (*Flaviviridae*) Dengue virus (DENV), Japanese encephalitis virus (JEV), and West Nile virus (WNV). The vector competence of *Ae. notoscriptus* was studied with BFV,[13,14] CHIKV,[15] RRV,[16,17] and DENV.[13,18] *Culex quinquefasciatus* vector competence was examined with MVEV,[19,20] RRV,[14,17,20,21] DENV,[22] JEV,[23] and WNV.[24] Competence of *Ae. australis* for SINV was studied with mosquitoes from Australia,[21,25] whereas NZ mosquitoes were used to study competence for RRV and DENV.[12] In addition, a theoretical risk assessment of the threat of CHIKV to NZ was published, although no experimental studies were conducted.[26] It is known that although the one endemic arbovirus in NZ, the alphavirus WHAV, is highly transmitted by *Ae. australis*,[2] and it readily infects birds, there has been no evidence of a confirmed human infection.[4]

The objective of the studies reported here is primarily to determine the ability of mosquitoes active in NZ to serve as competent vectors of viruses that might be introduced from other countries. In addition, we wanted to determine if an introduced virus would be able to survive the winter in resident mosquitoes, either through vertical transmission from

* Address correspondence to Laura D. Kramer, 5668 State Farm Road, Slingerlands, New York 12159. E-mail: LDK02@health.state.ny.us

parent to progeny through the eggs, or in overwintering adult females.

MATERIALS AND METHODS

Mosquitoes. The mosquitoes used in these studies (Table 1) include both endemic and introduced species to NZ, and colonized mosquitoes from North and Central America that served as controls. New Zealand mosquitoes were shipped to the Arbovirus Laboratories, Wadsworth Center as either eggs or larvae. The general rearing protocol (Table 2) consisted of larval rearing at 24°C in 35.6 cm × 27.9 cm × 8.3 cm plastic flats (Sterilite, Townsend, MA) containing 1 L of filtered water, and feeding every other day with koi food mixture, composed of a 1:1:1 ratio mixture of ground koi food (Kaytee products, Clinton, WI), lyophilized bovine liver powder (MP Biomedicals, Solon, OH), and rabbit chow (Kaytee fortified diet) with a light/dark cycle 16:8 hr. New Zealand adult mosquitoes generally were maintained at 24°C with the same light/dark cycle with 10% sucrose ad lib in 30.5 cm cages, with the exception of *Ae. antipodeus*, which were reared and maintained at 16°C, and the United States control colonies reared at 26°C but maintained at 24°C following infectious blood meal. Modifications to the standard protocol were guided by published literature on each species and adapted from knowledge of local breeding habitats. These modifications were tested in the laboratory and are noted below for each species. Before experimental vector competence assays were undertaken, all species were tested for blood meal preference and the preferred feeding hour used in all experimental work (Table 2).

Endemic species. *Opifex fuscus.* The eggs were collected in Wellington, NZ, and shipped to the Arbovirus Laboratory where a colony was established, guided by published procedures.[6] Larvae were reared in 75% salt water (Instant Ocean, Foster & Smith, Inc., Rhinelander, WI) diluted with 25% dechlorinated water. Mating of this species occurs on the water surface[27]; therefore, pupae were transferred to a 30.5cm^3 cage with a flat pan containing 75% salt water where the adults emerged and mated. Newly emerged females are autogenous; thus, for experimental feeding they were provided with cotton coils soaked in fresh water for an oviposition substrate 3 days before the infectious blood meal. The eggs were stored in a plastic bag and held in a covered container with a damp sponge at 15°C until needed.

Culex pervigilans. Larvae were collected from sites on both the North and South Islands and reared in low densities with dechlorinated water under the standard rearing protocol. Adults were maintained as described previously. A colony could not be established because eggs were never laid despite successful feeding by females on defibrinated bovine blood (Hema-Resources, Aurora, OR) at night.[28]

Aedes antipodeus. Larvae collected from the North Island, were shipped at various instar stages and reared at 16°C in low densities with a 7:17(L:D) cycle to simulate winter conditions. Emerged adults were maintained as described previously but held at 16°C. Because of the low rearing temperature, time from egg hatch to adult lasted ~30 d. A colony was not established because of low numbers available.

Introduced species. *Aedes australis.* Larvae, collected and shipped at various instar stages from Otago, were reared in 75% salt water (Instant Ocean) under the same general rearing protocol as described previously. A colony could not be established because the adult females refused to feed on blood from different sources.

*Culex quinquefasciatus **(NZ)**.* Larvae, collected and shipped at various instar stages from the Hutt Valley, were reared in dechlorinated water under the general rearing protocol described previously. Adults were fed weekly on defibrinated goose blood (Rockland Immunochemical Inc., Gilbertsville, PA) sweetened with 10% sucrose and provided with oviposition cups for egg rafts. A colony was successfully established.

Aedes notoscriptus. Eggs collected from Auckland were hatched and reared and adults were maintained as described under general protocols. A colony was not established because the necessary environmental conditions could not be met.

Control mosquitoes. All control species were maintained with water pads and sugar cubes in 30.5 cm cages and held at 26°C ± 2, with 70% ± 10 relative humidity with a photoperiod 16:8 (L:D).

*Aedes aegypti **(Panama)**.* Eggs were collected in Panama by J. Loaiza and sent to the Arbovirus Laboratory, Wadsworth Center, in 2007 to establish a colony. Larvae were reared

TABLE 1
Collection of mosquito species used in vector competence studies

Mosquito species	NZ*	Collections			
		Location	GPS coordinates	Dates	Stage†
Opifex fuscus	E	Island Bay, Wellington	–41°21′02.713″, 174°45′52.67″	07/07	Egg
		Moa Point, Wellington	–41°20′39.37″, 174°84′42.66″	07/07	Egg
Culex pervigilans	E	Kaipara Harbour, North Auckland	–36°26′46.14″, 174°25′18.22″	10/07, 11/07, 02/08, 09/08	Larvae
		Dunedin, Otago	–45°55′35.70″, 170°23′42.23″	03/08	Larvae
		Queenstown, Otago	–45°01′19.57″, 168°43′57.33″	02/08, 04/08	Larvae
Aedes antipodeus	E	Kaipara Harbour, North Auckland	–36°38′58.71″, 174°24′14.29″	05/09–09/09	Larvae
Aedes notoscriptus	I	Devonport, North Shore Auckland	–36°49′56.75″, 174°47′08.67″	12/08–03/09	Egg
		Kauri Point, North Shore Auckland	–36°49′24.31″, 174°41′53.81″	12/08–03/09	Egg
Aedes australis	I	Shag Point, Otago	–45°28′22.09″, 170°49′48.70″	12/09–03/10	Larvae
Culex quinquefasciatus (NZ)	I	Upper Hutt, Wellington	–41°07′55.35″, 175°03′21.56″	03/09, 09/09	Larvae
Culex pipiens (USA)	C	PA	N/A‡	N/A	N/A
Culex quinquefasciatus (USA)	C	Rutgers, NJ	N/A	N/A	N/A
Aedes albopictus	C	Japan	N/A	N/A	N/A
Aedes aegypti	C	Panama	N/A	N/A	N/A

* New Zealand (NZ) Endemic (E); NZ Introduced (I); non-NZ control (C) mosquito species.
† Developmental stage at time of collection.
‡ Not applicable.
GPS = global positioning system.

TABLE 2

Feeding and incubation of mosquito species used in vector competence studies

Mosquito species	Blood source*	Feeding time	Rearing temp	EI temp†
Opifex fuscus	Goose	Dawn	24°	24°C
Culex pervigilans	Bovine	Night	24°	24°C
Aedes antipodeus	Bovine	Dawn	16°	16°C
Aedes notoscriptus	Bovine	Dawn	24°	24°C
Aedes australis	Goose	Dawn	24°	24°C
Culex quinquefasciatus (NZ)	Goose	Dawn	24°	24°C
Culex pipiens	Goose	Dawn	26°	24°C
Culex quinquefasciatus (USA)	Goose	Dawn	26°	24°C
Aedes albopictus	Rabbit	Dawn	26°	24°C
Aedes aegypti	Rabbit	Dawn	26°	24°C

*Fed by pledget in all cases except *Cx. pervigilans* that would only feed on sausage casing with bovine blood.
†Extrinsic incubation temperature.

TABLE 3

Viruses evaluated in vector competence studies

Family, genus	Virus*	Strain	Origin	Source†
Togaviridae, Alphavirus	BFV	K10521	Mosquito (Australia)	UTMB
	CHIKV	91064A	Human (India)	CDC
	RRV	SW38457	Australia	UTMB
	SINV	Unknown	Unknown	UC Davis
Flaviviridae, Flavivirus	DENV-2	New Guinea C	New Guinea	Unknown
	DENV-2	98AO 1426	Thailand	Alan Rothman
	JEV	Nakayama	Japan	CDC
	MVEV	MK6684	Australia	UTMB
	WNV	02-1956	*Corvus brachyrhynchos* (NY, NY)	Wadsworth
	YFV	Be AR 645693	Mosquito (Brazil)	UTMB

*RRV = Ross River virus; BFV = Barmah Forest virus; SINV = Sindbis virus; CHIKV = Chikungunya virus; WNV = West Nile virus; DENV = Dengue virus; MVEV = Murray Valley encephalitis virus; JEV = Japanese encephalitis virus; YFV = Yellow fever virus.
†UTMB = University of Texas Medical Branch; CDC = Centers for Disease Control and Prevention; UC Davis = University of California, Davis.

and adults maintained as described previously. Females were fed weekly on defibrinated rabbit blood (Hema-Resources) sweetened with 10% sucrose. Eggs were collected weekly, allowed to embryonate, and stored in a small plastic bag in a closed container containing a moist sponge for humidity. When needed, the eggs were hatched under vacuum and larvae reared in low densities.

Aedes albopictus. Eggs were kindly provided in April 2007 by D. Severs en, Notre Dame University. Mosquitoes were reared and maintained in a colony, and eggs stored as described for *Ae. aegypti*.

Culex pipiens (USA). A colony was established from mosquitoes collected by M. Hutchinson in 2002 in Pennsylvania. Mosquitoes were reared and adults maintained as described previously except mosquitoes were maintained in 46 cm^3 cages. Adult females were fed weekly on defibrinated goose blood (Rockland Immunochemical) sweetened with 10% sucrose.

Culex quinquefasciatus (USA). Mosquito larvae were provided by D. Fonseca, Rutgers University in 2008. The colony originated from Benzon Research, Inc. (Carlisle, PA) who obtained the mosquitoes from Virginia Polytechnic Institute and State University. The adults were maintained at 26°C as described previously.

Mosquito feeding preferences. Blood preference and optimum feeding time were determined before the vector competence assays for each mosquito species (Table 2). A variety of defibrinated bloods were offered including guinea pig, sheep, rabbit, bovine, and goose using pledgets, sausage casing (porcine casing rinsed in the brine solution, knotted at one end, filled with the virus suspension, knotted, and warmed in a 30°C water bath), and membranes. The results (Table 2) guided the subsequent feedings.

Cells and viruses. *Aedes albopictus* cells (C6/36; ATCC CRL-1660) were grown in minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 2 mM L-glutamine, 0.3% sodium bicarbonate, 100 U of penicillin/mL, and 100 µg of streptomycin/mL and 1 U of non-essential amino acids/mL. African Green monkey kidney cells (Vero; ATCC CC1-81) were grown in the same medium but without non-essential amino acids.

The history of the 12 virus strains used in this study are listed in Table 3. The viruses included five flaviviruses (*Flaviviridae*, *Flavivirus*): WNV, MVEV, DENV serotype 2 (DENV2), Yellow fever virus (YFV), and JEV; and four alphaviruses (*Togaviridae*, *Alphavirus*) BFV, RRV, CHIKV, and SINV. Viruses used in the vector competence assays were generally prepared on the day of use from a fresh harvest of infected C6/36 cells; however, several studies used virus thawed from a frozen stock with a known concentration of virus, as noted in Tables 4 and 5.

Vector competence assays. *Peroral infection.* All work with infectious blood meals was conducted in the BSL3 insectary at the Arbovirus Laboratory, Wadsworth Center, New York State Department of Health, Albany, New York. Blood meals containing virus from frozen stocks were prepared by combining 4.5 mL of defibrinated goose, rabbit, or bovine blood, 2.5% (w/v) sucrose, 100 µL of adenosine 5′-triphosphate (ATP, 0.02 µM), and ~100 µL of virus stock. To increase virus titers and the efficiency of infection, most blood meals were prepared from freshly harvested virus-infected cells. Confluent C6/36 cell monolayers in 75 cm^2 flasks were infected at a multiplicity of infection of 0.1 plaque forming unit (pfu)/cell, and incubated at 28°C for 2–4 d, depending on the virus. The blood meal was prepared by scraping infected cells into the media, and mixing the suspension 1:1 with defibrinated blood plus ATP and 2.5% (w/v) sucrose.

Adult mosquitoes were mechanically aspirated from 30.5 cm^3 cages, placed in 4 L waxed cardboard cartons, and maintained with 10% sucrose for up to 1 week before vector competence studies. Before experimental feeding, mosquitoes were starved of sucrose for 24–36 hr. Peroral infection was achieved by exposing mosquitoes to blood meal-soaked pledgets for ~1 hr at room temperature, as previously described.[29] Exceptions were made for *Cx. pervigilans*, where the blood meal was offered in sausage casing overnight; and for *Ae antipodeus* that were starved 36–72 hr during which time they were slowly warmed to 24°C for feeding. In all cases, a 0.5 mL aliquot of the blood meal was taken both before and after the mosquitoes fed, and frozen at –80°C for subsequent plaque assay. After feeding, the mosquitoes were sorted under CO_2 and the fully engorged females were placed in 0.57 L cardboard containers. Fed females were maintained with sugar and moisture at 24°C.

After 7 and 14 d, or 21 and 30 d for *Ae antipodeus* because of the low 16°C incubation temperature, females were anesthetized with Triethylamine (Sigma, St. Louis, MO) for capillary

TABLE 4

Vector competence of New Zealand (NZ) mosquitoes for select alphaviruses (*Togaviridae*)

Mosq species and virus†	Blood meal‡ (log_{10} pfu/mL)	EIP§ (days)	% Infect¶ (no. tested)	% Dissem‖ (no. infect)	% Transm** (no. infect)
New Zealand mosquitoes					
Culex pervigilans					
BFV	6.4	20	0 (35)	–	–
		31	0 (10)	–	–
CHIKV	8.4	20	0 (40)	–	–
RRV	7.7	17	22 (23)	100 (5)	20 (5)
Opifex fuscus					
BFV	9.7	7	88 (8)	100 (7)	13 (7)
CHIKV	6.2	11	98 (47)	100 (46)	46 (46)
RRV	7.7	11	100 (19)	100 (19)	46 (19)
SINV	5.9	8	94 (31)	48 (29)	11 (29)
	6.0*	11	29 (35)	30 (10)	4 (10)
Aedes antipodeus					
BFV	10.0	21	92 (25)	100 (23)	43 (23)
CHIKV	7.8	21	100 (15)	73 (15)	0 (15)
RRV	8.4	21	82 (22)	89 (18)	61 (18)
		28	69 (16)	100 (11)	55 (11)
Aedes australis					
BFV	9.8	7	96 (25)	84 (24)	0 (24)
		14	100 (25)	92 (25)	44 (25)
RRV	8.7	7	96 (25)	75 (24)	4 (24)
		14	96 (25)	92 (24)	46 (24)
SINV	8.6	14	14 (35)	80 (5)	0 (5)
Aedes notoscriptus					
BFV	10.1	14	91 (11)	80 (10)	10 (10)
CHIKV	10.5	14	36 (22)	75 (8)	0 (8)
Culex quinquefasciatus (NZ)					
BFV	8.2	14	22 (50)	27 (11)	18 (11)
		30	12 (50)	33 (2)	17 (1)
RRV	8.6	14	3 (36)	100 (1)	100 (1)
SINV	6.7	7	0 (25)	–	–
		14	12 (25)	–	–
		21	24 (25)	50(3)	17(1)
Control mosquitoes					
Aedes aegypti (Panama)					
CHIKV	8.4	20	19 (47)	67 (9)	0 (9)
Culex pipiens (USA)					
BFV	6.4	8	2 (50)	0 (1)	–
RRV	7.7	16	15 (27)	0 (4)	–
SINV	6.0	11	8 (48)	50 (4)	25 (4)
Culex quinquefasciatus (USA)					
BFV	8.2	14	6 (49)	100 (3)	33 (3)
		30	6 (36)	100 (2)	0 (2)
RRV	8.6	14	6 (50)	100 (3)	0 (3)

†BFV = Barmah Forest virus; CHIKV = Chikungunya virus; RRV = Ross River virus; SINV = Sindbis virus.
‡All blood meals were freshly harvested cell culture virus unless noted with an asterisk (*) indicating frozen stock.
§Extrinsic incubation period at 24°C except *Ae. antipodeus*, which were held at 16°C
¶Percentage (number) of blooded mosquitoes with virus-positive bodies.
‖Percentage (number) of infected mosquitoes with virus-positive legs.
**Percentage (number) of infected mosquitoes with virus-positive expectorates.

transmission assays as described previously.[29] Salivary secretions, legs, and bodies were harvested and frozen separately at –80°C for subsequent determination of transmission, dissemination, and infection rates, respectively. Body and leg samples were prepared for virus titration by homogenization using a mixer mill (Qiagen, Valencia CA) at 24 cycles/sec for 30 sec, and then clarified by centrifugation at 10,000 rpm for 1 min.

Long-term survival of infected mosquitoes was tested where number of specimens received allowed. In some cases, low numbers of larvae collected in NZ made assessment of vertical transmission impossible because vector competence studies were considered a priority. The mosquitoes were infected as described, held for extended periods of time up to 120 d, and then tested for ability to transmit.

Vertical transmission. Vertical transmission studies could be conducted only with mosquitoes that would mate and lay fertile eggs successfully, allowing these studies to be conducted with two species, *Op. fuscus* and *Cx. quinquefasciatus*. Female and male adult *Op. fuscus m*osquitoes were aspirated from the 30.5cm^3 cage after 1 week to allow mating, and placed in 4 L cardboard cartons, and provided with cotton coils soaked in fresh water for an oviposition substrate. After the females had laid the first batch of autogenous eggs, the mosquitoes were deprived of sugar and water for 18–24 hr, and then fed a blood meal containing RRV. Engorged females were placed in 0.57 L cardboard containers in a 24°C incubator with a 16:8 (L:D) cycle. Cotton coils were checked daily for eggs. Once eggs appeared, the cotton coil was changed and the eggs were placed in a plastic bag labeled with date and oviposition cycle. Eggs were subsequently submerged in 75% salt water, allowed to hatch, and reared to the fourth instar stage, which was harvested for subsequent detection of virus. Larvae were pooled for assay in groups of five because of the large size of the larvae, rinsed sequentially with dechlorinated water, phosphate buffered saline (PBS[-]), and 1.0 mL of mosquito diluent (MD; PBS[-] supplemented with 20% fetal bovine serum and antibiotics), and then frozen in fresh 1.0 mL MD at –80°C for subsequent titration by plaque assay. Parental females were frozen at –80°C at the termination of the experiment and processed as described previously to determine overall infection rate, although eggs were collected en masse and individual adults were not distinguished.

Culex quinquefasciatus females were allowed to mate with males for 1 week, then fed blood meals containing WNV, and incubated as for *Op. fuscus*. Once a week, an oviposition cup with filtered water was introduced into the carton through a sleeve in the container while working in a secure plexiglass sleeve box. After removal of the egg rafts, the mosquitoes were offered a fresh uninfected blood meal. The egg rafts were allowed to hatch and larvae were fed a koi food mixture daily. Fourth instar larvae were harvested, as described previously, and frozen at –80°C in pools of 10–25 for subsequent virus testing. Larvae and adults harvested from the vertical transmission studies were tested for presence of WNV RNA by reverse transcription-polymerase chain reaction (RT-PCR).

Virus assays. *Plaque assays.* Virus titrations were performed by inoculating 100 µL of the salivary secretions or supernatant of homogenized bodies and legs onto confluent monolayers of Vero cells in 6-well Costar plates (Corning Life Sciences, Lowell, MA). Larval pools from the vertical transmission assay with *Op. fuscus* were homogenized in pools of five and 100 µL were inoculated onto confluent monolayers of Vero cells. Plaque assay was used for the larval pools rather than RT-PCR because the latter assay was not developed for use with RRV in the Arbovirus Laboratory. Plaque assays were conducted as described previously[30] to determine presence of infectious virus in the mosquito specimens. Titers of the blood meal harvested before and after the mosquitoes fed were also determined by plaque assay.

Real-time RT-PCR. Mosquito adults and larvae from the vertical transmission assays were tested for the presence of WNV RNA by real-time RT-PCR.[31] If viral RNA was detected, plaque assay for infectious virus was planned. Samples were homogenized as described for vector competence assays, and RNA was extracted from 350 µL of clarified homogenates

TABLE 5
Vector competence of New Zealand (NZ) mosquitoes for select flaviviruses (*Flaviviridae*)

Mosquito species and virus†	Blood meal titer‡ (log_{10} pfu/mL)	EIP§ (days)	% Infect¶ (no. tested)	% Dissem‖ (no. infect)	% Transm** (no. infect)
New Zealand mosquitoes					
Culex pervigilans					
DENV-2 Thai	6.7	23	48 (48)	9 (23)	4 (23)
MVEV	6.7	21‖	0 (25)	–	–
WNV	9.4	21	47 (60)	14 (28)	7 (28)
Opifex fuscus					
DENV-2 Thai	5.7	11	56 (50)	11 (28)	0 (28)
JEV	8.1	14	74 (50)	70 (37)	0 (37)
MVEV	7.4	7–11	48 (104)	40 (50)	0 (50)
	6.9*	11	0 (39)	–	–
WNV	7.4	7–11	63 (38)	54 (24)	0 (12)
	6.9*	10	0 (13)	–	–
YFV	5.5	14	54 (50)	0 (27)	–
Aedes antipodeus					
DENV-2 NGC	5.6	21	100 (16)	56 (16)	25 (16)
WNV	10.4	21	7 (15)	0 (1)	–
		43	5 (19)	0 (1)	–
Aedes australis					
DENV-2 NGC	6.9	7	4 (25)	100 (1)	0 (0)
MVEV	8.1	14	13 (15)	50 (2)	0 (2)
WNV	8.8	7	32 (25)	63 (8)	25 (8)
Aedes notoscriptus					
DENV-2 NGC	6.7	14	4 (27)	0 (1)	–
JEV	8.3	14	0 (39)	–	–
MVEV	8.5	14	0 (25)	–	–
		28	0 (29)	–	–
WNV	8.1	15	7 (15)	100 (1)	0 (0)
Culex quinquefasciatus (NZ)					
JEV	8.1	14	17 (36)	0 (6)	–
MVEV	8.7	14	33 (39)	31 (13)	8 (13)
		30	43 (28)	33 (12)	17 (12)
WNV	9.6	60	100 (20)	45 (20)	20 (20)
Control mosquitoes					
Aedes aegypti (Panama)					
DENV-2 NGC	6.7	14	20 (25)	20 (5)	0 (5)
DENV-2 Thai	5.0	11	28 (50)	7 (28)	0 (28)
YFV	5.5	30	13 (30)	25 (4)	0 (4)
Aedes albopictus					
DENV-2 Thai	5.7	30	82 (28)	96 (23)	5 (23)
Culex pipiens (USA)					
JEV	8.1	14	10 (50)	40 (5)	0 (5)
MVEV	6.9	30	72 (32)	91 (23)	9 (23)
WNV	8.9	15	96 (50)	54 (48)	4 (48)
	6.9*	14	40 (50)	45 (20)	20 (20)
Culex quinquefasciatus (USA)					
JEV	8.1	14	86 (50)	0 (43)	0 (43)
MVEV	8.7	14	2 (50)	100 (1)	100 (1)

† DENV-2 = Dengue virus 2, Thai or New Guinea C strains; JEV = Japanese encephalitis virus; MVEV = Murray Valley encephalitis virus: WNV = West Nile virus; YFV = Yellow fever virus.
‡ All blood meals were freshly harvested cell culture virus unless noted with an asterisk (*) indicating frozen stock.
§ Extrinsic incubation period at 24°C, except *Ae antipodeus*, which were held at 16°C.
¶ Percentage (number) of blooded mosquitoes with virus-positive bodies.
‖ Percentage (number) of infected mosquitoes with virus-positive legs.
** Percentage (number) of infected mosquitoes with virus-positive expectorates.

using RNeasy kits (Qiagen). The assay was performed on an ABI Prism 7500 PCR system, using TaqMan One-Step RT-PCR master mix (Applied Biosystems, Foster City, CA) according to manufacturer's protocol. The 50 µL reaction consisted of 1 µM of each primer pair, 0.2 µM of probe, and 10 µL of extracted RNA. The sequences of the primer/probe set specific for the WNV NS5 region, were 5′-GCTCCGCTGTCCCTG TGA-3′, 5′-CACTCTCCTCCTGCATGGATG-3′, and 5′-6-carboxyfluorescein-TGGGTCCCTACCGGAAGAACCA CGT- 6-carbox-N,N,N',N'-tetramethylrhodamine -3′. Ten-fold dilutions of WNV (800–0.08 pfu) were used as standards in every assay. Thermal cycling consisted of 48°C for 30 min for reverse transcription, 95°C for 10 min, and 40 cycles of 95°C for 15 sec, and 60°C for 1 min. Data were analyzed by identifying the amplification cycle at which fluorescence increased above threshold (Ct), which was fixed at 0.1. A sample was determined to be positive if the Ct value was equal to or less than the threshold Ct value.

ID_{50} studies. Viruses were amplified on C6/36 cells and harvested from cells on the day of infections, as described for peroral infections. To determine the dose at which 50% of mosquitoes became infected (ID_{50}), blood meals containing 10-fold serial dilutions of harvested virus were prepared and used to feed 100–200 females/dose. Engorged females were maintained at 24°C with a 16:8 (L: D) cycle for 7–14 d, and then frozen for subsequent virus plaque assay of the original blood meal and homogenized mosquitoes. The ID_{50} of each strain was calculated by the method of Reed and Muench.[32]

RESULTS

Peroral vector competence of endemic species. *Culex pervigilans* was a competent vector of RRV (*Alphavirus*) and WNV (*Flavivirus*) following feeding on high but not unrealistic titers of virus (Tables 4 and 5, respectively). This species was not a competent vector of BFV (*Alphavirus*) or MVEV (*Flavivirus*) following feeding on a moderate dose, or of CHIKV (*Alphavirus*) following feeding on a high dose. It is surprising that *Cx. pervigilans* were able to become infected with and transmit DENV after 23 d extrinsic incubation because most *Culex* species generally are not considered vectors of this virus as a consequence of their ornithophilic nature.

Opifex fuscus was a competent vector of all five alphaviruses tested, but did not transmit any of the six flaviviruses tested even though mosquitoes became infected and virus disseminated from the midgut. Transmission might have occurred were the females longer lived. Mosquitoes were significantly more efficiently infected after feeding on freshly harvested virus compared with frozen virus stock. This was observed with WNV and MVEV, although titers of the frozen stock were 0.5 $\log_{10}$ pfu lower, and with SINV where the blood meals contained the same concentration of virus.

Aedes antipodeus was a competent vector for the alphaviruses BFV and RRV, but did not transmit CHIKV after 21 d extrinsic incubation; this may have been a consequence of the low temperature of incubation, 16°C, since virus disseminated well from the midgut. This species also was a poor host for WNV, with only 7% infection after 21 d and no mosquitoes infected on Day 43, even after feeding on 10.4 $\log_{10}$ pfu virus. However, despite the low temperature of extrinsic incubation, this species was a highly competent DENV vector.

Peroral vector competence of introduced species. The two NZ introduced *Aede*s species tested were competent vectors for most alphaviruses examined (Table 4). Although *Ae. notoscriptus* did not transmit CHIKV after 14–21 d, it was surprising that the control colony, *Ae. aegypti*, also did not transmit virus, possibly because of the 24°C incubation. It should be noted that we have not had problems working with CHIKV in the past. *Aedes australis* was poorly infected with SINV. The NZ *Cx. quinquefasciatus* mosquitoes became infected and transmitted BFV and RRV, but the levels were low. Similar low levels were observed with *Cx. pipiens* and *Cx. quinquefasciatus* from the United States and with the NZ endemic *Cx. pervigilans*. The NZ *Cx. quinquefasciatus* mosquitoes were poor vectors for SINV, although transmission was achieved after 21 d extrinsic incubation.

The NZ introduced mosquito species did not prove to be competent vectors for flaviviruses, except for WNV, which infected and was transmitted well by *Ae. australis* and *Cx. quinquefasciatus*, and MVEV, which *Cx. quinquefasciatus* transmitted at a low level (Table 5).

Vertical transmission. Two attempts at vertical transmission with *Op. fuscus* after successful feeding on a blood meal containing 8 $\log_{10}$ pfu/mL of RRV led to negative results. In one experiment, viable eggs were obtained from 120 RRV-fed females after the second ovarian cycle (OV2), yielding a final number of 31 pools of five larvae. The third ovarian cycle (OV3) yielded two pools of five larvae each, both negative. The second experiment yielded 100 OV2 larvae from 50 RRV-fed females, which were tested in pools of five with negative results. Additional attempts were unsuccessful because no additional parental females survived to OV2. All OV2 eggs were laid ~4–5 d after infectious blood meal, which most likely was too early for transmission to occur efficiently at 24°C. The numbers of OV3 larvae laid on Day 10 are too low to draw conclusions. It is expected that all parental mosquitoes were infected because the 50% infectious dose of RRV for *Op. fuscus* is 4.3 $\log_{10}$ pfu/mL (Table 6).

No vertical transmission was detected for WNV in *Cx. quinquefasciatus*. Two experiments were performed by successfully feeding a total of 182 mosquitoes on blood meals containing 9 $\log_{10}$ pfu/mL of WNV. The total number of OV1 progeny tested was 1,190. A second ovarian cycle was completed, with a total of 300 progeny tested, all of which were negative. Seventeen parental females tested for virus infection were positive. The 50% infectious dose of WNV for *Cx. quinquefasciatus* is 8.4 $\log_{10}$ pfu/mL (Table 6).

ID_{50} determination. The ability of endemic and introduced NZ mosquito species to become infected by arboviruses also was evaluated where numbers of mosquitoes allowed, by calculating the dose at which 50% of mosquitoes became infected (ID_{50}) (Table 6). *Opifex fuscus* was highly susceptible to RRV, BFV, YFV, and DENV-2 with ID_{50} values of 4.3, 5.4, 5.5, and 5.7, respectively. This species also demonstrated good infection by the other four viruses tested. Generally, at the low NZ temperatures used, TD_{50} values (concentration of virus fed upon that led to 50% of the mosquitoes transmitting virus) could not be determined precisely, therefore these data are not presented. *Aedes australis* were less susceptible to BFV than *Op. fuscus*; and *Cx. pervigilans* were less susceptible to DENV and WNV than *Op. fuscus*. Similarly, *Cx. quinquefasciatus* were less susceptible to WNV than *Op. fuscus*.

Survival in *Cx. quinquefasciatus* for extended periods of time (Table 7). Long-term survival of infected *Cx. quinquefasciatus* was conducted to determine survivorship and ability to transmit virus after extended incubation at winter temperatures. The mosquitoes were infected as described, then maintained at 10°C for 60–120 d after infection with WNV or JEV before being tested for infection and ability to transmit virus. After 60 and 90 d, 100% of *Cx. quinquefasciatus* were infected, and 20% and 10%, respectively, were able to transmit WNV at 10°C. This is significant as it indicates this

TABLE 6
ID_{50} of New Zealand mosquito species with selected arboviruses

Mosquito	Virus*	Log_{10} ID_{50}†
Aedes australis	BFV	7.3
Culex pervigilans	DENV-2 Thai	6.7
	WNV	9.4
Culex quinquefasciatus	WNV	8.4
Opifex fuscus	BFV	5.4
	CHIKV	< 6.2
	RRV	4.3
	SINV	7.7
	DENV-2 Thai	5.7
	MVEV	7.4
	WNV	< 7.4
	YFV	5.5

*BFV = Barmah Forest virus; RRV = Ross River virus; DENV-2 = Dengue-2 virus; WNV = West Nile virus; MVEV = Murray Valley encephalitis virus; CHIKV = Chikungunya virus; SINV = Sindbis virus; YFV = Yellow fever virus.
†50% infectious dose. Blood meal titer in $\log_{10}$ pfu/mL.

Table 7

Vector competence of New Zealand *Culex quinquefasciatus* maintained at 10°C for an extended incubation period

Virus*	Blood meal titer (log_{10} pfu/mL)	EIP† (days)	% Infect‡ (no tested)	% Dissem§ (no infect)	% Transm¶ (no infect)
WNV	9.6	60	100 (20)	45 (20)	20 (20)
		90	100 (10)	40 (10)	10 (10)
JEV	8.1	120	6 (16)	100 (1)	0 (0)

*WNV = West Nile virus; JEV = Japanese encephalitis virus.
† Extrinsic incubation period.
‡ Percentage (number) of blooded mosquitoes with virus-positive bodies.
§ Percentage (number) of infected mosquitoes with virus-positive legs.
¶ Percentage (number) of infected mosquitoes with virus-positive expectorate.

species is capable of acting as a viral reservoir for WNV over the winter months. The JEV infection of *Cx. quinquefasciatus* decreased from 74% at Day 14 to 6% after 120 d at 10°C, and no mosquitoes were able to transmit virus.

DISCUSSION

The mosquitoes chosen for this study were the most likely available mosquitoes of NZ's limited mosquito fauna to be potential vectors of arboviruses to people and animals because of their abundance, distribution, and host preferences. It was observed that the species evaluated have the potential to be efficient vectors of most of the alphaviruses examined, if they were to be introduced into the country. However, they are less competent vectors of flaviviruses, most likely a result of the cool temperatures of incubation, typical of the NZ environment, used in these studies. Flaviviruses replicate faster at warmer temperatures; thus, although the mosquitoes were susceptible to the flaviviruses tested, they were unable to complete the extrinsic incubation period to allow transmission to occur successfully. This was particularly true for *Op. fuscus*, a highly susceptible mosquito, but very short-lived, thereby preventing completion of the extrinsic incubation period at 24°C. Nonetheless, this species was highly competent for CHIKV, a virus that presents a current threat to the country as it is expanding rapidly in Asia.[26] Other studies with CHIKV and Australian mosquitoes have been conducted, demonstrating the threat this virus poses to regions with competent vectors.[15,33] Vector competence experiments evaluating CHIKV containing the alanine to valine mutation first observed in La Reunion[34,35] should be conducted to determine whether there is a difference in competence for this new genotype that is currently circulating so widely in nearby regions.

The observation that freshly harvested virus led to more efficient infection than frozen stock of the same virus has been observed previously with DENV by others.[36,37] Those results were confirmed with WNV, MVEV, and SINV in this study.

Published results from Australia evaluating vector competence of *Ae. notoscriptus*[23] showed higher levels of competence for JEV (27% infection, 8% dissemination, 27% transmission) than this study demonstrated; however, mosquitoes were incubated at 28°C, and therefore cannot be directly compared with the results presented here where the NZ population of this mosquito was incubated at lower temperatures more appropriate for NZ. Results obtained for BFV were similar to those obtained here. *Culex quinquefasciatus* from Australia had 56% infection and 0% dissemination for JEV at 28°C, which was similar to our results at 24°C;[23] and similar results were also obtained for RRV and MVEV.

Despite the low extrinsic incubation temperature simulating winter conditions (16°C), *Ae. antipodeus* were efficiently infected with BFV, RRV, and DENV-2, and were able to transmit virus after 21 d, and possibly earlier. This species, therefore, should be monitored for BFV, RRV, and DENV infection.

It was not determined whether the alphaviruses tested would survive the cool winters because insufficient numbers of mosquitoes were available to achieve colonization to conduct such studies. However, survival of WNV and JEV over an extended period of time at 10°C was observed. *Culex quinquefasciatus* were able to transmit WNV after ≥ 90 d at 10°C, thus potentially allowing the virus to survive through winter. The JEV was found in a low number of mosquitoes of this same species held for 120 d, but the numbers were too low to conclude this would not be a possible mechanism of overwintering of this virus. This mosquito species is not known to transmit vertically at high rates, as we confirmed in this study. Further studies should be conducted to determine the potential for vertical transmission by *Aedes* species mosquitoes of selected alphaviruses. *Opifex fuscus* have short life spans at temperatures favorable for arboviral replication, probably too short to allow efficient infection of ovaries for vertical transmission to be successful. However, because females of this species will live more than 30 d at 18°C, vertical transmission may be possible at lower temperatures with longer periods of incubation.

The results of this comprehensive study indicate that NZ mosquito species are competent vectors of some arboviruses of medical importance, particularly alphaviruses. However, factors such as feeding preferences, population density, and survivorship also need to be taken into consideration in assessing risk. Therefore, additional field studies should be conducted to determine the conditions under which the laboratory competent species present a risk. Surveillance activities are critical to NZ's biosecurity and should be conducted for selected arboviruses, particularly CHIKV, DENV, MVE, and RRV because of the high level of activity in the neighboring regions.[38] A regional approach to control mosquito-borne viruses has been wisely advocated because of the high risk to this geographic area populated with immunologically naive individuals.[26] Although no endemic activity of arboviral pathogens that infect humans is evident in NZ now, climate change may increase the probability of invasive viruses becoming established.

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Authors' addresses: Laura Kramer, Pan Chin, and Elizabeth Kauffman, Wadsworth Center, New York State Department of Health, Slingerlands, New York and (Kramer) School of Public Health, Department of Biomedical Sciences, State University of New York at Albany, Albany, NY Slingerlands, New York, E-mails: LDK02@health.state.ny.us, pac18@health.state.ny.us, and ebk01@health.state.ny.us. Rachel Cane, Mosquito Consulting Services (NZ) Lincoln,

New Zealand, E-mail: rachel.cane@mcspty.com. Graham Mackereth, ESR NCBID-Wallaceville, Upper Hutt, New Zealand, E-mail: gra ham.mackereth@esr.cri.nz.

REFERENCES

1. Miles JA, Ross RW, Austin FJ, Maguire T, Macnamara FN, Ross LM, 1971. Infection of wild birds with Whataroa virus in South Westland, New Zealand, 1964–1969. *Aust J Exp Biol Med Sci 49:* 365–376.
2. Miles JA, 1973. The ecology of Whataroa virus, an alphavirus, in South Westland, New Zealand. *J Hyg (Lond) 71:* 701–713.
3. Maguire T, Miles JA, Casals J, 1967. Whataroa virus, a group A arbovirus isolated in South Westland, New Zealand. *Am J Trop Med Hyg 16:* 371–373.
4. Derraik JG, 2004. Exotic mosquitoes in New Zealand: a review of species intercepted, their pathways and ports of entry. *Aust NZ J Public Health 28:* 433–444.
5. Weinstein P, Laird M, Browne G, 1997. *Exotic and Endemic Mosquitoes in New Zealand as Potential Arbovirus Vectors*. Wellington, NZ: Ministry of Health, 1–16.
6. Haeger JS, Provost MW, 1965. Colonization and biology of *Opifex fuscus*. *Trans R Soc NZ 6:* 21–108.
7. Belkin JN, 1968. Mosquito studies (Diptera: Culicidae). VII. The Culicidae of New Zealand. *Contrib Am Entomol Inst 3:* 1–182.
8. Derraik JG, 2005. Aussie mozzies invade NZ. *Australian Science 26:* 23–25.
9. Nye ER, 1962. *Aedes* (*Pseudoskusea*) *australis* Erichson (Diptera, Culicidae) in New Zealand. *Trans R Soc NZ 3:* 33–34.
10. Laird M, 1994. *Aedes notoscriptus* (Diptera: Culididae) in Wellington Province 135. *NZ Entomol 17:* 14–17.
11. Laird M, 1990. New Zealand's northern mosquito survey, 1988–89. *J Am Mosq Control Assoc 6:* 287–299.
12. Maguire T, 1994. Do Ross River and dengue viruses pose a threat to New Zealand? *NZ Med J 107:* 448–450.
13. Watson TM, Kay BH, 1999. Vector competence of *Aedes notoscriptus* (Diptera: Culicidae) for Barmah Forest virus and of this species and *Aedes aegypti* (Diptera: Culicidae) for dengue 1–4 viruses in Queensland, Australia. *J Med Entomol 36:* 508–514.
14. Kay BH, Boyd AM, Ryan PA, Hall RA, 2007. Mosquito feeding patterns and natural infection of vertebrates with Ross River and Barmah Forest viruses in Brisbane, Australia. *Am J Trop Med Hyg 76:* 417–423.
15. van den Hurk AF, Hall-Mendelin S, Pyke AT, Smith GA, and MacKenzie JS, 2009. Vector competence of Australian mosquitoes for Chikungunya virus. *Vector Borne Zoonotic Dis 10:* 489–495.
16. Watson TM, Kay BH, 1998. Vector competence of *Aedes notoscriptus* (Diptera: Culicidae) for Ross River virus in Queensland, Australia. *J Med Entomol 35:* 104–106.
17. Russell RC, 2002. Ross River virus: ecology and distribution. *Annu Rev Entomol 47:* 1–31.
18. Knox TB, Kay BH, Hall RA, Ryan PA, 2003. Enhanced vector competence of *Aedes aegypti* (Diptera: Culicidae) from the Torres Strait compared with mainland Australia for dengue 2 and 4 viruses. *J Med Entomol 40:* 950–956.
19. Kay BH, Fanning ID, Mottram P, 1989. Rearing temperature influences flavivirus vector competence of mosquitoes. *Med Vet Entomol 3:* 415–422.
20. Kay BH, Fanning ID, Carley JG, 1982. Vector competence of *Culex pipiens quinquefasciatus* for Murray Valley encephalitis, Kunjin, and Ross River viruses from Australia. *Am J Trop Med Hyg 31:* 844–848.
21. Dhileepan K, Azuolas JK, Gibson CA, 1996. Evidence of vertical transmission of Ross River and Sindbis viruses (*Togaviridae*: Alphavirus) by mosquitoes (Diptera: Culicidae) in southeastern Australia. *J Med Entomol 33:* 180–182.
22. Vazeille-Falcoz M, Rosen L, Mousson L, Rodhain F, 1999. Replication of dengue type 2 virus in *Culex quinquefasciatus* (Diptera: Culicidae). *Am J Trop Med Hyg 60:* 319–321.
23. van den Hurk AF, Nisbet DJ, Hall RA, Kay BH, MacKenzie JS, Ritchie SA, 2003. Vector competence of Australian mosquitoes (Diptera: Culicidae) for Japanese encephalitis virus. *J Med Entomol 40:* 82–90.
24. Jansen CC, Webb CE, Northill JA, Ritchie SA, Russell RC, van Den Hurk AF, 2008. Vector competence of Australian mosquito species for a North American strain of West Nile virus. *Vector Borne Zoonotic Dis 8:* 805–811.
25. Ovenden JR, Mahon RJ, 1984. Venereal transmission of Sindbis virus between individuals of *Aedes australis* (Diptera: Culicidae). *J Med Entomol 21:* 292–295.
26. Derraik JG, Slaney D, Nye ER, Weinstein P, 2010. Chikungunya virus: a novel and potentially serious threat to New Zealand and the South Pacific islands. *Am J Trop Med Hyg 83:* 755–759.
27. Provost MW, Haeger JS, 1967. Mating and pupal attendance in Deinocerites cancer and comparisons with *Opifex fuscus* (Diptera: Culicidae). *Ann Entomol Soc Am 60:* 565–574.
28. Graham DH, 1939. Mosquito life in the Auckland District. *Trans Proc R Soc NZ 69:* 210–224.
29. Ebel GD, Carricaburu J, Young D, Bernard KA, Kramer LD, 2004. Genetic and phenotypic variation of West Nile virus in New York, 2000–2003. *Am J Trop Med Hyg 71:* 493–500.
30. Payne AF, Binduga-Gajewska I, Kauffman EB, Kramer LD, 2006. Quantitation of flaviviruses by fluorescent focus assay. *J Virol Methods 134:* 183–187.
31. Kauffman EB, Jones SA, Dupuis AP, Ngo KA, Bernard KA, Kramer LD, 2003. Virus detection protocols for West Nile virus in vertebrate and mosquito specimens. *J Clin Microbiol 41:* 3661–3667.
32. Reed LJ, Muench H, 1938. A simple method of estimating fifty per cent endpoints. *Am J Hyg 27:* 493–497.
33. Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, Rocha BC, Hall-Mendelin S, Day A, Riegler M, Hugo LE, Johnson KN, Kay BH, McGraw EA, van Den Hurk AF, Ryan PA, O'Neill SL, 2009. A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and *Plasmodium*. *Cell 139:* 1268–1278.
34. Vazeille M, Moutailler S, Coudrier D, Rousseaux C, Khun H, Huerre M, Thiria J, Dehecq JS, Fontenille D, Schuffenecker I, Despres P, Failloux AB, 2007. Two chikungunya isolates from the outbreak of La Reunion (Indian Ocean) exhibit different patterns of infection in the mosquito, *Aedes albopictus*. *PLoS ONE 2:* e1168.
35. Tsetsarkin KA, Vanlandingham DL, McGee CE, Higgs S, 2007. A single mutation in chikungunya virus affects vector specificity and epidemic potential. *PLoS Pathog 3:* e201.
36. Miller BR, 1987. Increased yellow fever virus infection and dissemination rates in *Aedes aegypti* mosquitoes orally exposed to freshly grown virus. *Trans R Soc Trop Med Hyg 81:* 1011–1012.
37. Richards SL, Pesko K, Alto BW, Mores CN, 2007. Reduced infection in mosquitoes exposed to blood meals containing previously frozen flaviviruses. *Virus Res 129:* 224–227.
38. Derraik JG, Calisher CH, 2004. Is New Zealand prepared to deal with arboviral diseases? *Aust NZ J Public Health 28:* 27–31.