Larval Nutritional Stress Does Not Affect Vector Competence for West Nile Virus (WNV) in *Culex tarsalis*

Brittany L. Dodson,^{1,2} Laura D. Kramer,^{2,3} and Jason L. Rasgon^{1,4}

Abstract

In some mosquito species the conditions experienced by larvae during development have been shown to lead to changes in susceptibility to various arboviruses in the adult female. Since laboratory mosquitoes are generally reared under ideal conditions, mosquito vector competence experiments in the laboratory may not accurately reflect vector–virus relationships in nature. We examined the consequences of larval nutritional stress on *Culex tarsalis* vector competence for West Nile virus (WNV). Larval nutrition deprivation resulted in increased development time, decreased pupation and emergence rates, and smaller adult female body size. However, infection, dissemination, and transmission rates for WNV at 5, 7, and 14 days postfeeding were not consistently affected. These results suggest that larval nutritional rearing protocols are not a major factor in laboratory estimates of WNV vector competence in *Cx. tarsalis*.

Key Words: *Culex*—West Nile.

Introduction

THE FRESHWATER MOSQUITO, *Culex tarsalis*, is generally associated with areas used for agriculture, as well as artificial containers in urban areas (Bohart and Washino 1978). In the field, mosquito larvae may be subjected to unstable or inadequate ecological conditions, including nutritional deprivation, temperature changes, poor water quality, and competition for food, which can have consequences on larval development and survival (Reisen et al. 1984, Agnew et al. 2000). The fitness of adult mosquitoes is influenced by their larval environment. Multiple studies have shown that stress during larval development can lead to significantly smaller females and can potentially affect mosquito susceptibility to pathogens, including arboviruses (Grimstad and Haramis 1984, Alto et al. 2008, Westbrook et al. 2010). Intra- and interspecific competition studies with *Aedes aegypti* and *Aedes albopictus* showed that smaller females were more likely to have disseminated dengue-2 infections (Alto et al. 2008). Nutritional deprivation of *Aedes triseriatus* larvae produced small adults that transmitted *La Crosse virus* more efficiently than adults from well-fed larvae (Grimstad and Haramis 1984). However, other studies found that large *Aedes* were

more likely than the small females to be infected with *Dengue-2 virus* (Sumanochitrapon et al. 1998) and *Chikungunya virus* (Westbrook et al. 2010). No correlation between *Cx. tarsalis* body size and vector competence for *Western equine encephalitis virus* (WEEV) and *St. Louis encephalitis virus* (SLEV) was observed (Reisen et al. 1997); however, nutrition was not a variable in these studies.

Mosquitoes used in laboratory experiments are raised under ideal conditions and are generally larger than those found in the field (Mather and DeFoliart 1983). If the larval rearing environment affects the biology of the adult insect, there is the unexplored possibility that laboratory mosquito vector competence experiments may not accurately reflect vector–virus relationships in nature if mosquito-rearing conditions are not taken into account. The majority of previous work examining the effects of environmental stress has focused on *Aedes* mosquitoes. However, *Culex* species also serve as competent vectors for numerous arboviruses, including West Nile virus (WNV), WEEV, SLEV, Eastern equine encephalitis virus, and Ross River virus. *Cx. tarsalis* is a major vector of WEEV and SLEV in much of the western United States (Bohart and Washino 1978) and is one of the most competent laboratory vectors for WNV (Goddard et al. 2002). In the present study,

¹The W. Harry Feinstone Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland.

²Arbovirus Laboratories, Wadsworth Center, New York State Department of Health, Slingerlands, New York.

³School of Public Health, State University of New York at Albany, Albany, New York.

⁴The Johns Hopkins Malaria Research Institute, Baltimore, Maryland.

we examined whether nutritional deprivation during larval development affected vector competence of *Cx. tarsalis* for WNV. We also evaluated how larval nutrition status affects overall development of this mosquito species.

Materials and Methods

Mosquitoes, cells, and virus

Cx. tarsalis obtained from a colony at the Wadsworth Center Arbovirus Laboratories were used for all experiments. The colony was established from egg rafts collected in the Coachella Valley, CA, in 2003. Colonized mosquitoes were reared and maintained at $27^{\circ}\text{C} \pm 1^{\circ}\text{C}$, with a 16:8 h light:dark photoperiod at ~45% ambient relative humidity with additional humidity added directly by moist toweling. Larvae were fed a 1:1:1 ratio of ground koi food, ground rabbit pellets, and bovine liver powder (MP Biomedicals, CA). Adults were allowed access to 10% sucrose solution *ad libitum* through a cotton wick and were provided goose blood through a membrane feeder for egg development.

African green monkey kidney (Vero) cells were used for virus titrations and screening of mosquitoes. *Ae. albopictus* (C636) cells were used to amplify virus at 37°C and 5% CO_2 . WNV strain WN02-1956 (Gen Bank accession number AY590222) was used for all experiments. The strain was derived from the kidney of an infected American crow in 2003 and was originally isolated in Vero cells. After one round of amplification in C636 cells, the titer of the virus stock was $9.5\text{E}+9$ PFU/mL. All experiments with infectious WNV were completed in the Wadsworth Center, Arbovirus Laboratories' BSL-3 insectary, and laboratory facilities according to established biosafety protocols.

Larval nutritional treatments

Three separate replicate experiments were performed, each utilizing two larval nutritional treatments (ideal or stressed). For each experiment, 300 newly hatched first-instar larvae were placed into $35.6 \times 27.9 \times 8.3$ cm plastic pans (Sterilite, MA) containing 1 L of filtered water, with three to seven pans per treatment (Table 1). Food was added to each pan 6 days per week. Feeding regimes for ideal and stressed treatments were empirically developed in preliminary experiments where the stressed rearing protocol was the least amount of food that could be given without compromising eventual development into adults—reducing food levels beyond this point made it logistically impossible to consistently rear enough mosquitoes for experiments. At each feeding, larvae in the ideal (control) treatment group were given the following food amounts (per pan): 60 mg for first and second instar larvae, 90 mg for third instar, and 180 mg for fourth instar. Larvae undergoing nutritional stress were given one-third the amount of food as those in the control treatment group.

Pupae in each treatment were placed in 50 mL conical tubes filled with distilled water. After emergence, adults were removed to 3.8 L cartons and held at 27°C until blood feeding. The number of pupae, the number of males, and number of females were recorded daily. Mean initial time to pupation, initial time to adulthood, percent pupation, and percent emergence were determined and the two treatments compared using *t*-tests.

TABLE 1. EFFECT OF NUTRITIONAL STRESS ON *Cx. tarsalis* LARVAL REARING PARAMETERS

Treatment type	Exp 1	Exp 2	Exp 3
Days to first pupation ^a			
Ideal	7.3 (7)	8 (3)	8 (4)
Stressed	10.1 (7)	10 (3)	10.4 (5)
<i>p</i> value	<0.0001	<0.0001	<0.0001
Days to first emergence ^a			
Ideal	10	10	10
Stressed	11.7	11.3	12.4
<i>p</i> value	<0.0001	0.008	<0.0001
% pupation ^b			
Ideal	91	78	81
Stressed	72	59	75
<i>p</i> value	<0.0001	<0.0001	0.0001
% pupal emergence ^b			
Ideal	92	85	93
Stressed	76	86	89
<i>p</i> value	<0.0001	0.5410	0.0003
% emergence larvae to adult ^b			
Ideal	83	66	76
Stressed	55	51	67
<i>p</i> value	<0.0001	<0.0001	<0.0001
Adult winglength (mm) ^a			
Ideal	3.28	3.35	3.37
Stressed	3.11	3.06	3.04
<i>p</i> value	<0.0001	<0.0001	<0.0001

Values in parentheses indicate number of pans per experiment.

^a*t*-test.

^b χ^2 -test.

Vector competence

Seven- to 10-day-old mosquitoes were starved for 24 h before feeding. WNV strain WN02-1956 was added to 5 mL defibrinated goose blood (Rockland, PA) with 2.5% sucrose to an approximate titer of 10^9 PFU/mL. The final titers for the control group in experiments one, two, and three were 9.2, 9.0, and 8.8 $\log_{10}$ PFU/mL and the titers for the stressed group were 8.9, 9.1, and 8.8 $\log_{10}$ PFU/mL. Mosquitoes were fed via a Hemotek membrane feeder (Discovery Workshops, Accrington, UK) for ~1 h. After feeding, engorged females were removed to new 3.8 L cartons under CO_2 anesthesia, provided with 10% sucrose *ad lib*, and held for up to 14 days at 27°C , 16:8 light:dark photoperiod.

At 5, 7, and 14 days postfeeding, transmission assays were performed as described (Aitken 1977) with slight modifications. Briefly, female mosquitoes were anesthetized with triethylamine (Sigma, St. Louis, MO) and all legs removed into 1 mL mosquito diluent (MD; 20% heat-inactivated fetal bovine serum in Dulbecco's phosphate-buffered saline plus 50 $\mu\text{g}/\text{mL}$ penicillin/streptomycin, 50 $\mu\text{g}/\text{mL}$ gentamicin, and 2.5 $\mu\text{g}/\text{mL}$ fungizone). The proboscis of each mosquito was placed in a capillary tube containing 10 μL of a 1:1 solution of 50% sucrose and fetal bovine serum. After 30 min, contents of the capillary tube were expelled into 0.3 mL MD. One wing per mosquito was removed for subsequent mea-

surement as an indicator of body size. Bodies were placed into 1 mL MD, and all samples were stored at -70°C until processed. Mosquito bodies and legs were homogenized in a mixer mill (Qiagen) and clarified by brief centrifugation. Bodies, legs and salivary secretions were screened via Vero plaque assay as previously described (Payne et al. 2006) for presence of infectious virus. Infection was defined as the proportion of mosquitoes with infected bodies. Dissemination and transmission were defined as the proportion of infected mosquitoes with infected legs and salivary secretions, respectively. Data were analyzed using Fisher's exact test.

Mosquito wings were measured from the alular notch to the distal margin, excluding the fringe using Axiovision software and Zeiss microscope according to the manufacturer's specifications. Differences in wing length between mosquitoes in the two nutritional treatment groups were compared using *t*-test.

Results

Mosquito development

Development time, numbers of immature mosquitoes at various stages, and adult body size were determined to examine the potential consequences of nutritional stress (Table 1) on *Cx. tarsalis* development. Unsurprisingly, larvae reared under ideal nutritional conditions had significantly better developmental parameters than larvae reared under nutritionally stressed conditions. Larvae reared under the ideal protocol pupated significantly faster, had a higher pupation rate, emerged faster and at a higher rate, and resulted in larger adults as estimated by wing length (Table 1). In general, results between experimental replicates were not statistically different.

Vector competence

To evaluate the effects of nutritional stress on *Cx. tarsalis* vector competence for WNV, we examined infection, dissemination, and transmission of WNV at 5, 7, and 14 days postfeeding (dpf). In experiment 1, the infection rates among the nutritionally stressed mosquitoes were significantly lower than the control group at 7 and 14 dpf (Table 2). However, this observation was not repeatable in the other two experimental replicates (Table 2). Except for experimental replicate 2, where stressed mosquitoes had a higher dissemination rate than nonstressed at 7 dpf, no other significant differences in dissemination or transmission were observed between treatments and timepoints (Table 2).

Discussion

Knowledge of the effects of environmental conditions on immature mosquito development is important for placing the results of laboratory experiments on mosquito biology and vector competence into the context of natural conditions, since the fitness of adults can be affected by their larval environment (Hardy et al. 1983). *Cx. tarsalis* is an important species epidemiologically because they are found in most freshwater environments, have catholic feeding habits (Bohart and Washino 1978), are one of the most competent vectors for WNV (Goddard et al. 2002) and SLEV (Meyer et al. 1983) in the laboratory, and have been implicated as important vector species in the field (Reisen et al. 2004, Bell et al. 2005, Barker et al. 2009, Venkatesan and Rasgon 2010). We studied the

TABLE 2. EFFECT OF LARVAL NUTRITIONAL STRESS ON ADULT *Cx. tarsalis* VECTOR COMPETENCE FOR WEST NILE VIRUS

Treatment type	Day postfeeding	Exp 1 ^a	Exp 2 ^b	Exp 3 ^b
% Infected				
Ideal	5	72	76	88
Stressed		44	72	80
<i>p</i> value ^c		0.085	0.820	0.414
Ideal	7	80	68	88
Stressed		44	80	90
<i>p</i> value ^c		0.019	0.254	1.000
Ideal	14	92	72	88
Stressed		48	82	82
<i>p</i> value ^c		0.002	0.342	0.577
% Disseminated ^d				
Ideal	5	27.8	18.4	43.2
Stressed		63.6	22.2	22.5
<i>p</i> value ^c		0.119	0.776	0.064
Ideal	7	70.0	38.2	77.3
Stressed		72.7	65.0	71.1
<i>p</i> value ^c		1.000	0.035	0.630
Ideal	14	91.3	77.8	84.1
Stressed		91.7	85.4	95.1
<i>p</i> value ^c		1.000	0.555	0.158
% Transmitted ^e				
Ideal	5	0.0	28.6	15.8
Stressed		0.0	25.0	0.0
<i>p</i> value ^c		1.000	1.000	0.530
Ideal	7	7.1	30.8	20.6
Stressed		12.5	19.2	28.1
<i>p</i> value ^c		1.000	0.689	0.570
Ideal	14	71.4	35.7	70.3
Stressed		54.6	60.0	66.7
<i>p</i> value ^c		0.442	0.077	0.808

^aN=25 for each timepoint.

^bN=50 for each timepoint.

^cFisher's exact test.

^dCalculated based on number of infected mosquitoes.

^eCalculated based on number of mosquitoes with disseminated infection.

Italicized, bold *p* values represent significant results.

effects of nutritional deprivation at the larval stage on immature *Cx. tarsalis* development and vector competence of adults for WNV. Our results indicated that although nutritional deprivation had a significant effect on immature development, it did not have a consistent effect on vector competence.

In the present study, pupation and emergence of the nutritionally deprived larvae was delayed and reduced compared to the control group. Similar impacts on immature *Cx. tarsalis* development and survival have previously been observed with other stressors, such as altering rearing temperature and larval density (Reisen et al. 1984). Stressed larvae also developed into smaller adults. The nutritional status of mosquito larvae during development also has been found to correlate with adult body size in *Ae. triseriatus* and *Cx. quinquefasciatus* (Grimstad and Haramis 1984, Agnew et al. 2000).

Studies with several *Aedes* species have observed that larval stress affects vector competence, correlating female mosquito body size with susceptibility to arboviruses. Small *Ae. triseriatus*

females were more susceptible to La Crosse virus than large females (Grimstad and Haramis 1984, Paulson and Hawley 1991). Larval competition studies with *Ae. aegypti* and *Ae. albopictus* produced small females that were more likely to have disseminated DENV-2 infections (Alto et al. 2008). Correlations between large *Aedes* females and higher susceptibility to DENV-2, Ross River virus, and Chikungunya virus have also been found (Nasci and Mitchell 1994, Sumanochitrapon et al. 1998, Westbrook et al. 2010).

We did not observe consistent differences in infection, dissemination, or transmission rates between the control and nutritionally stressed mosquitoes. It previously has been shown that larval nutritional deprivation does not significantly affect the vector competence of adult *Cx. tritaeniorhynchus* for WNV or *Cx. annulirostris* for Murray Valley encephalitis virus (Baqar et al. 1980, Kay et al. 1989). An additional study of Japanese encephalitis virus in *Cx. tritaeniorhynchus* observed a slight difference in patterns of transmission depending on larval nutritional status (Takahashi 1976). However, these data are confounded by the different ages of the nutritionally deprived and control mosquitoes at the time of blood feeding, as it is known that adult age can affect vector competence (Baqar et al. 1980, Richards et al. 2009). The results of studies on other environmental stressors were similar. Alterations in the rearing temperature of *Cx. pipiens* larvae did not affect adult susceptibility to Rift Valley fever virus, and changes in the quality of larval rearing water affected adult *Cx. tarsalis* body size, but not vector competence for WEEV and SLEV (Brubaker and Turell 1998, Reisen et al. 1997). Our data, in combination with previous studies, suggest that environmental stress during larval development has little to no effect on the vector competence of *Culex* species. It is interesting to speculate that changes in the larval environment appear to affect *Aedes* species more severely than *Culex*, especially in virus susceptibility and possibly adult morphology.

Our study highlights the importance of studying the effects of the mosquito larval environment on arbovirus–vector interactions. Our data suggest that larval nutritional stress is not an important factor in laboratory evaluations of *Cx. tarsalis* vector competence for WNV. However, we only investigated one out of many potential larval stressors. Future experiments will explore the potential effects of other larval environmental conditions on *Cx. tarsalis* development and competence for WNV.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Jason L. Rasgon

The W. Harry Feinstone Department of Molecular

Microbiology and Immunology

Bloomberg School of Public Health

Johns Hopkins University

Baltimore, MD 21205

E-mail: jrasgon@jhsp.edu