Quantitative Genetics of Vector Competence for La Crosse Virus and Body Size in *Ochlerotatus hendersoni* and *Ochlerotatus triseriatus* Interspecific Hybrids

Justin R. Anderson, Jennifer R. Schneider, Paul R. Grimstad and David W. Severson[1]

Center for Tropical Disease Research and Training, Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana 46556

ABSTRACT

La Crosse virus is a leading cause of pediatric encephalitis in the United States. The mosquito *Ochlerotatus triseriatus* is an efficient vector for La Crosse virus, whereas the closely related *O. hendersoni* transmits only at very low rates. Quantitative trait loci (QTL) affecting the ability to orally transmit this virus and adult body size were identified in 164 F_2 female individuals from interspecific crosses of *O. hendersoni* females and *O. triseriatus* males using a combination of composite interval mapping (CIM), interval mapping (IM) for binary traits, and single-marker mapping. For oral transmission (OT), no genome locations exceeded the 95% experimentwise threshold for declaring a QTL using IM, but single-marker analysis identified four independent regions significantly associated with OT that we considered as tentative QTL. With two QTL, an increase in OT was associated with alleles from the refractory vector, *O. hendersoni*, and likely reflect epistatic interactions between genes that were uncovered by our interspecific crosses. For body size, two QTL were identified using CIM and a third tentative QTL was identified using single-marker analysis. The genome regions associated with body size also contain three QTL controlling OT, suggesting that these regions contain either single genes with pleiotropic effects or multiple linked genes independently determining each trait.

L A Crosse virus (LACV; family Bunyaviridae, genus Bunyavirus, species California encephalitis virus) is a leading cause of pediatric encephalitis in the United States, with ~70 cases reported annually (Centers for Disease Control and Prevention 2003), and is distributed throughout the eastern United States (Calisher 1983). *Ochlerotatus triseriatus*, the Eastern treehole mosquito, is an efficient vector of LACV (Watts *et al.* 1972); however, the closely related *O. hendersoni* transmits only rarely, at frequencies of <10% (Grimstad *et al.* 1985; Paulson *et al.* 1989). Nontransmitting *O. hendersoni* develop a disseminated infection, and LACV readily infects and replicates in the salivary glands. This suggests that the failure to pass virus orally is due to inability of the virus to escape from the salivary glands. Concurrent infection with both LACV and the avian malaria parasite, *Plasmodium gallinaceum*, results in bypassing this barrier, and transmission rates are similar to those seen in *O. triseriatus* (Paulson *et al.* 1992). Hence, the inability to transmit by *O. hendersoni* is likely due to a physical barrier that prevents escape of the virus; coinfection with the metazoan parasite results in disruption of this barrier and subsequent transmission of the virus.

Vectorial capacity refers to the ability of an intermediate (vector) host to transfer a pathogen between susceptible vertebrate hosts. After a mosquito takes a viremic blood meal, the virus must successfully passage several potential morphological and physiological barriers to be transmitted by the mosquito to another vertebrate host. Many factors, both intrinsic and extrinsic, can affect the success of this transmission. Significant extrinsic factors include temperature, humidity, and nutrition. At higher temperatures, dissemination is more rapid and viral titers rise more quickly with most mosquito/virus systems investigated to date (Bates and Roca-García 1946; Chamberlain and Sudia 1955; Kay *et al.* 1989; Cornel *et al.* 1993; Reisen *et al.* 1993). However, the opposite effect is observed with some mosquito/virus systems (Reisen *et al.* 1993), and results in modulated or even cleared infections (Kramer *et al.* 1983). Exposure to high humidity has been shown to both enhance dengue virus infections and increase daily probability of survival in *Aedes aegypti* (Thu *et al.* 1998). Nutritional status of adult *Anopheles stephensi* (Koella and Sorensen 2002) and larval *O. triseriatus* (Grimstad and Haramis 1984) has been shown to significantly impact vectorial capacity.

Vector competence, as a critical component of vectorial capacity, is determined by intrinsic factors that predispose the innate immune response of a mosquito vector to a pathogen (Beerntsen *et al.* 2000). In some

Anopheles/Plasmodium systems, malarial parasites are killed by melanotic encapsulation, thus preventing their transmission (COLLINS *et al.* 1986), as are larvae of the filarioid nematode parasite, *Brugia malayi*, by *Armigeres subalbatus* (FERDIG *et al.* 1993). Incompatibilities between a refractory strain of *A. aegypti* and *P. gallinaceum* result in the near-complete failure of parasite development (THATHY *et al.* 1994), likely due to an immunity-mediated lytic reponse that seems to also be activated in most strains of *A. aegypti* when exposed to filarioid nematode parasites (MACDONALD 1962). Finally, several studies have observed that vector competence for arbovirus transmission varies among mosquito populations, including LACV by *O. triseriatus* (GRIMSTAD *et al.* 1977).

Quantitative trait loci (QTL) that control vector competence have been mapped with several mosquito/pathogen systems. ZHENG *et al.* (1997) mapped QTL that control melanotic encapsulation of several Plasmodium spp. by *Anopheles gambiae*. With *A. aegypti*, QTL have been mapped that influence both *P. gallinaceum* (SEVERSON *et al.* 1995; MEECE 2002) and *B. malayi* development (SEVERSON *et al.* 1994; BEERNTSEN *et al.* 1995), as well as for midgut infection and midgut escape barriers for dengue viruses (BOSIO *et al.* 2000). GRAHAM *et al.* (2003) mapped QTL determining transovarial transmission of LACV by *O. triseriatus*. However, the genetic basis for oral transmission of LACV or other arboviruses has not been investigated. In this study, we used progeny from interspecific crosses between *O. hendersoni* and *O. triseriatus* to map QTL for oral transmission (OT) of LACV. In addition, because recent studies have shown that body size in *A. aegypti* may be correlated with vector competence for *P. gallinaceum* (MEECE 2002) and dengue virus (J. R. SCHNEIDER and D. W. SEVERSON, unpublished data), we examined whether genome regions containing QTL for oral transmission of LACV might also correspond to those containing QTL for body size.

MATERIALS AND METHODS

Mosquito strains and rearing: *O. triseriatus* WALTON strain was collected as eggs in 1969 from northern Indiana and has been maintained in the laboratory for over 100 generations. *O. hendersoni* PEORIA strain was established from eggs collected in Peoria, Illinois. It was initiated and maintained by forced copulation (McDANIEL and HORSFALL 1957), and the F_3 generation was used for these studies. Mosquito colonies were reared in an environmental chamber at 21° and ~80% relative humidity as previously described (MUNSTERMANN and WASMUTH 1985). Adults were provided honey-soaked cotton as a sugar source. Female mosquitoes were blood fed on anesthetized mice. Interspecific crosses were established by force copulating *O. triseriatus* WALTON males to *O. hendersoni* PEORIA females 1–2 days after the females had fed on anesthetized mice, as this crossing scheme was previously shown to produce viable, fertile offspring (TAYLOR 1987). The F_1 progeny also were force copulated to establish an F_2 generation.

Infection of mosquitoes with LACV: Virus was obtained by inoculating a confluent layer of African green monkey kidney (Vero) cells (American Type Cell Culture, Manassas, VA) with

1 ml virus suspension from the previous passage and 1 ml M199 medium in 75-cm^2 tissue culture flasks at 37° and 5% CO_2 as previously described (GRIMSTAD and HARAMIS 1983, 1984). Adult female mosquitoes were orally infected with LACV (strain M18304A; 13 suckling mouse brain and 3 Vero cell passages) diluted to a titer of 4.97 log $TCID_{50}$ in defibrinated rabbit blood (Colorado Serum Company, Denver) via an artificial membrane feeder warmed to ~37° by a circulating water system following procedures of GRIMSTAD *et al.* (1977). All females were allowed to feed on the same infectious blood meal and only those that fully engorged were retained and provided honey as a sugar source until tested for OT.

Determination of oral transmission: Female mice [Hsd: ICR (CD-1)] with newborn litters were purchased from Harlan (Indianapolis) and maintained as family units in separate cages. Female mosquitoes were transferred individually to small cages (50-ml centrifuge tubes with fine mesh tops) and 2- to 3-day-old suckling mice were then gently hand held against the mesh until the mosquito had (1) fed to repletion, (2) probed three or more times, or (3) probed at least once, but showed no further interest. Because a single probing event is sufficient to transmit virus (GUBLER and ROSEN 1976; GRIMSTAD *et al.* 1977), even those females that probed only once but did not take a full blood meal were frozen at −80° until further assayed. In addition, a single wing from each female was removed and fixed to a glass slide to determine whether there was a correlation between body size and transmission. Wing lengths, as a proxy for body size, were measured from the apical notch to the axillary margin using an ocular micrometer. Because these species have previously been shown to differ significantly in OT (PAULSON *et al.* 1989) and wing length (*O. triseriatus* is larger than *O. hendersoni*; ZAVORTINK 1972), we did not obtain phenotype measurements from the parent strains. Also, due to the low success rate in interspecific matings and resulting small sample sizes (only a limited number of forced mating attempts are successful), phenotypes were not determined for F_1 females. Approximately one-half of the F_2 females were tested for OT 26 days postinfection (p.i.), while the remainder were tested on day 33 p.i. due to limited availability of suckling mice from the supplier. A single toe was clipped from each mouse as an identifier (DUBIN 1968). One mouse from each litter served as an uninfected control. Individual suckling mice fed on by individual mosquitoes were returned to cages with their mothers and observed for 14 days for signs of central nervous system disturbance; pups displaying signs were killed by rapid freezing at −80°.

Oral transmission was confirmed by plaque assay of aspirated brain material from individual frozen suckling mice in six-well tissue culture plates (Costar, Acton, MA) as described by GRIMSTAD *et al.* (1977). Following an initial 1-hr incubation of diluted brain tissue on Vero cells, the tissue homogenates were aspirated from the cell monolayer, and 2 ml M199, with 0.8% gum tragacanth, 25 μg/100 ml amphotericin B, and 15 μg/100 ml gentamycin, was added to each well. Plates were incubated for 3 days at 37° and 5% CO_2. Plaques were visualized by fixing and staining the plates for at least 1 hr in 0.1% crystal violet/10% formalin, rinsing in tap water, and air drying.

Midguts of mosquitoes were dissected and tested for the presence of LACV by plaque assay as described above. In addition, head squashes, followed by indirect immunofluorescence antibody tests (IFAs) were used to determine whether virus had disseminated (GUBLER and ROSEN 1976; GRIMSTAD and HARAMIS 1983). An uninfected head served as a negative control on each slide.

DNA extraction, PCR amplification, and genotyping: Genomic DNA was extracted from individual mosquitoes as previously described (SEVERSON 1997). PCR-based genetic marker

TABLE 1

Genetic markers used in *O. hendersoni* and *O. triseriatus* interspecific mapping

Marker	Accession nos.[a]	Type[b]	Restriction enzyme (species cut[c])
AeW[d]	BV096896, BV096919	SNP	*Rsa*I (T)
LF179[d]	BV096880, BV096904	SNP	*Hpa*II (T) or *Bam*HI (H)
AtL73	BV096890, BV096913	SNP	*Nla*III (T)
AtL07	BV096884, BV096908	SNP	*Hae*III (H)
AtL14	BV096886, BV096921	SNP	*Rsa*I (H)
LF159[d]	BV096879, BV096903	SNP	*Bam*HI (H)
LF284[d]	BV096881, BV096905	SNP	*Mbo*I (H)
AEGI8[d]	BV096892, BV096915	SNP	*Alu*I (H)
AtIAP	BV096893, BV096916	SNP	*Hha*I (T)
AtL26	BV096887, BV096910	SSCP	—
LF115	R67978	RFLP	—
AtL11	BV096885, BV096909	In/del	—
VCP[d]	BV096895, BV096918	SSCP	—
AtL42	BV096888, BV096911	SSCP	—
LF92[d]	BV096874, BV096898	SSCP	—
LF158[d]	BV096878, BV096902	SNP	*Hha*I (T)
LF111[d]	BV096876, BV096900	SNP	*Bst*UI (T)
LF99[d]	BV096875, BV096899	SNP	*Xsp*I (T)
LF296[d]	BV096882, BV096906	SSCP	—
AtL72	BV096889, BV096912	SNP	*Taq*I (T)
ATE001	BV096891, BV096914	SNP	*Mbo*II (T)
LF334[d]	BV096883, BV096907	SSCP	—
CRALBP[d]	BV096894, BV096917	SSCP	—
LF129[d]	BV096877, BV096901	SNP	*Nla*III (H)
PABP[d]	BV096897, BV096920	SSCP	—

[a] Accession numbers for *O. hendersoni* and *O. triseriatus*, respectively, are listed.

[b] SNP, single nucleotide polymorphism restriction digest; SSCP, single-strand conformation polymorphism on PAGE gels; In/del, 28-bp insertion in *O. hendersoni* sequence; RFLP, restriction fragment length polymorphism analysis of Southern blot of genomic DNA following methods of SEVERSON (1997).

[c] H, *O. hendersoni*; T, *O. triseriatus*.

[d] Comparative anchor-tagged sequences (CATS) based on *A. aegypti* cDNA sequences previously developed (CHAMBERS *et al.* 2003) or developed in this study.

loci were developed from two sources. First, comparative anchor-tagged sequences (CATS) identified from *A. aegypti* cDNA sequences were tested for PCR amplification with five individuals each from *O. triseriatus* and *O. hendersoni* as described by CHAMBERS *et al.* (2003). In addition, several new CATS were developed for this study, again using *A. aegypti* cDNA sequences selected on the basis of their genome location in *A. aegypti* (SEVERSON *et al.* 2002). Second, primer sequences for several markers derived from *O. triseriatus* cDNAs were generously provided by William C. Black of Colorado State University prior to publication (GRAHAM *et al.* 2003) or were derived from *O. triseriatus* sequences taken from GenBank. Primers were designed using the PRIMER program (Whitehead Institute, Cambridge, MA). Details on markers used in constructing an interspecific linkage map are given in Table 1.

Individual markers were scored as species-specific single nucleotide polymorphism (SNP) loci. To identify informative SNPs, the target sequence was amplified from up to five individuals per species and subjected to direct cycle sequencing using the ABI Prism Big Dye Terminator kit (Applied Biosystems, Foster City, CA). Sequences were aligned using the default settings of ClustalW on the BCM Searchlauncher website (http://searchlauncher.bcm.tmc.edu) and species-specific SNPs were identified visually. Whenever possible, SNP analysis

followed the McSNP protocol (AKEY *et al.* 2001). Species-specific SNPs located within restriction enzyme recognition sites were identified using the NEBcutter program (http://tools.neb.com/NEBcutter/index.php3). McSNP products from individual segregants were visualized and scored using a gel-free system (Hybaid DASH system; Thermo Hybaid, Franklin, MA). With one marker (AtL11), a diagnostic indel polymorphism was noted following sequence analysis, which allowed for visualization directly in 3% agarose gels. For markers where no restriction site was associated with the diagnostic SNP, the PCR products were visualized as single-strand conformation polymorphisms (SSCPs) on 5% polyacrylamide gels following standard protocols (FULTON *et al.* 2001; MEECE 2002).

Linkage and statistical analyses: A composite linkage map was constructed from four parental crosses; females from three of these crosses were examined in this study. Linkage distances were estimated using the MAPMAKER computer program (LANDER *et al.* 1987) with a LOD of 3.0 as the threshold for significance and were compared to an existing linkage map for *O. triseriatus* (ANDERSON *et al.* 2001). Map distances are reported in Kosambi centimorgans (KOSAMBI 1944).

Identification of QTL controlling OT was performed using QTL-by-SAS (XU and XU 2003); this program identifies QTL associated with binary traits by assuming an underlying continuous trait that results in the phenotype only when a threshold

is exceeded. Significance thresholds for declaring a QTL were estimated using the method of PIEPHO (2001). QTL locations were determined using the interval mapping (IM) for categorical traits module of QTL-by-SAS with a 1-cM step size. Linkage group (LG) III was evaluated as two linkage groups to remove any confounding effect of the large interval between markers ATE001 and LF334 (see Figure 1).

QTL controlling body size were identified using the QTL Cartographer computer package (BASTEN *et al.* 2001). Markers with significant partial regression coefficients were identified with the SRmapqtl function by forward-backward stepwise regression. Quantitative trait loci affecting each trait were identified by composite interval mapping (CIM; ZENG 1993, 1994) using the Zmapqtl function with model 6. An experimentwise likelihood ratio (LR) threshold for identifying a QTL was determined by permutation test (CHURCHILL and DOERGE 1994). The data were permuted 1000 times, and a critical LR value was determined for the $\alpha = 0.05$ significance level. LGIII was again evaluated as two linkage groups.

In addition to interval mapping methods for QTL analysis, we also used simple, single-marker tests to assess relationships between phenotype and marker genotype. These procedures are rigorous for identifying significant marker associations, but are inadequate for accurately defining QTL locations (DOERGE 2002). We included these analyses as (1) interval mapping has low power across large (>30 cM) intervals, (2) the experimentwise thresholds for interval mapping are conservative and likely to exclude valid QTL (CHURCHILL and DOERGE 1994), and (3) we were unable to determine linkage associations for one marker (*PABP*) and thus could not include it for interval mapping. Fisher's exact test using the FREQ procedure in the SAS computer package (SAS INSTITUTE 1990, 1997) was performed for OT. Significant associations with body size were identified using proc GENMOD in SAS. Markers at $P < 0.05$ were considered to have a significant association with the phenotype, while markers with $P < 0.10$ were considered as suggestive of an association. Adjustments for multiple tests (*e.g.*, Bonferroni corrections) were not performed for two reasons. First, linkage data, and, thus, QTL analyses, are likely not appropriate for these adjustments as markers on individual chromosomes are not independent, and multiple loci affecting a phenotype may not be independent even across chromosomes. Second, because of the relatively small number of unique marker tests involved in our study ($n = 20$), at the 5% significance level we expect only a single false positive.

RESULTS

Quantitative genetics of OT: A total of 164 female F_1 intercross progeny were obtained from three interspecific crosses between an *O. hendersoni* female and an *O. triseriatus* male that provided a sufficiently large number of progeny for genetic analyses; these families consisted of 32, 33, and 99 individual females, respectively. The positions of the 24 genetic markers and the sex locus mapped in 502 progeny from four pairwise crosses (this includes both males and females) are shown in Figure 1. We were unable to determine linkage associations for one marker (*PABP*); this was not surprising, as other markers from the homologous genome region in *A. aegypti* also remain unlinked in *O. triseriatus* (ANDERSON *et al.* 2001). As observed previously (ANDERSON *et al.* 2001), segregation ratios deviated from the expected
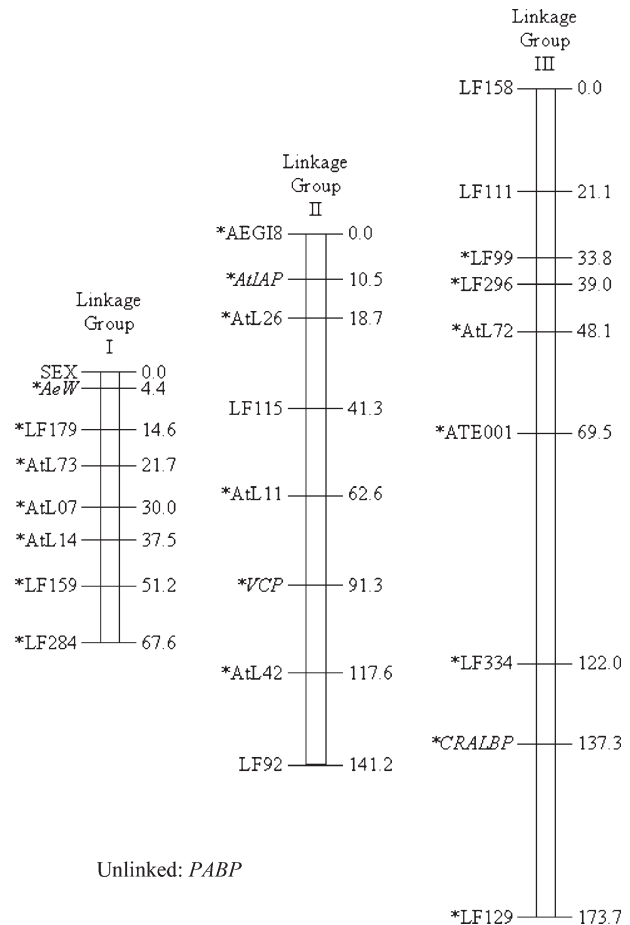

FIGURE 1.—Linkage map for 502 progeny of four *O. hendersoni* female by *O. triseriatus* male crosses. Genetic markers are given to the left of the chromosome; KOSAMBI (1944) centimorgan distances are to the right. Markers identified with an asterisk were used to map oral transmission and body size in 164 female progeny.

Mendelian ratio of 1:2:1 for all markers except *AtIAP* on LGII (data not shown). Markers identified with an asterisk in Figure 1 were used in this study.

Following OT trials, midguts were dissected to confirm LACV presence by plaque assay, while the heads were removed and tested for the presence of LACV antigen by IFA. Virus was present in 100% of the midguts, indicating that each female had taken an infective blood meal. Head squashes were inconclusive; there appeared to be very little specific fluorescence when compared to uninfected controls. Because OT was documented (see below), and because others have previously shown both *O. hendersoni* and *O. triseriatus* to readily disseminate virus upon infection (GRIMSTAD *et al.* 1985; PAULSON *et al.* 1989, 1992), we assumed that all females tested for OT had disseminated infections.

Due to difficulties obtaining sufficient suckling mice from the supplier, 66 female mosquitoes (approximately one-half from the two smallest families and the remainder from the largest) were tested 26 days p.i., and the remaining 98 were tested 33 days p.i. Transmis-
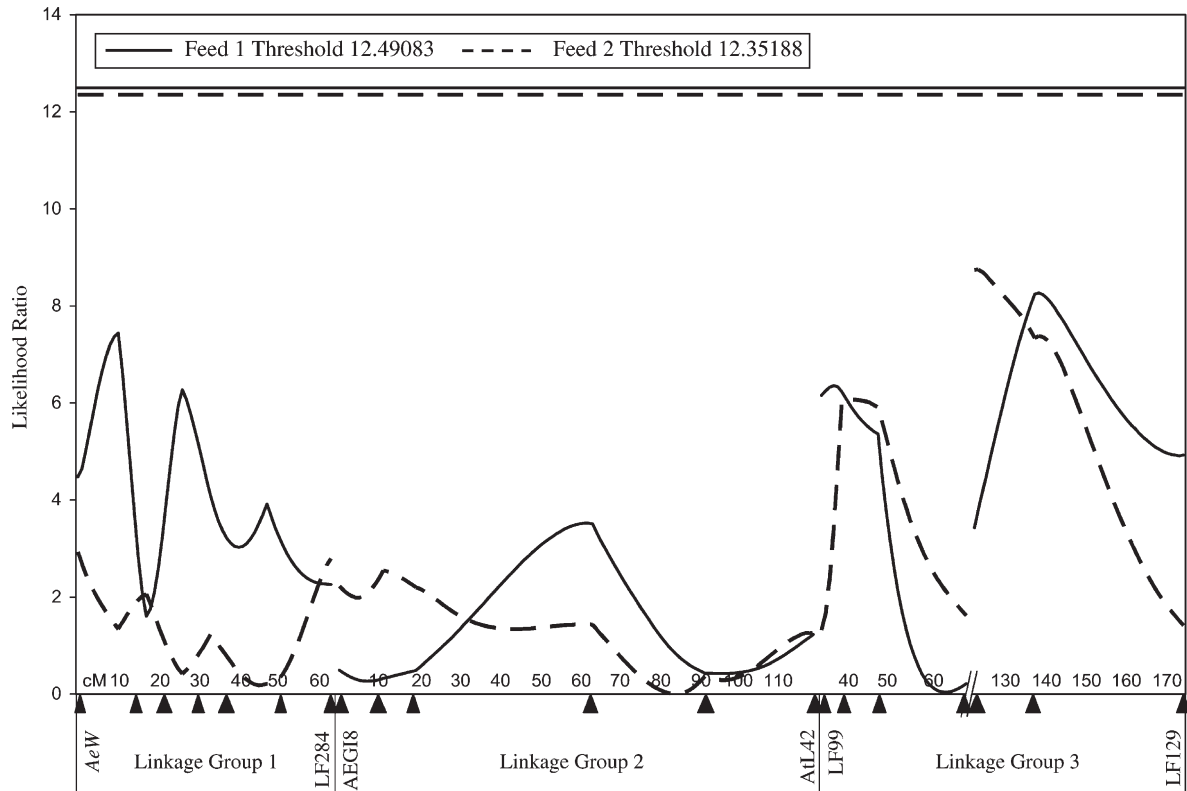
FIGURE 2.—Likelihood ratio profile for identifying quantitative trait loci for oral transmission of La Crosse virus. The solid line represents feeding group 1, while the dashed line represents feeding group 2. Horizontal lines are the experimentwise threshold value for identifying a QTL. Triangles represent the relative location of genetic markers (see Figure 1); two marker names are given for orientation of each chromosome.

sion rates between families and between feeding dates were compared by logistic regression. No significant differences in transmission rates were found between families (family 4, 35% transmitted; family 14, 18%; family 16, 25%; $P = 0.1479$). However, significant differences were found between feeding groups (FG1, 18% transmitted; FG2, 38%; $P = 0.0084$). Therefore, for subsequent analyses each feeding group was tested separately for QTL determining OT. No significant differences in transmission rates were seen with respect to the number of times females probed ($P = 0.54$ for FG1 and 0.73 for FG2) or whether females engorged ($P = 0.08$ for FG1 and 0.76 for FG2).

Forty-nine of 164 (29.9%) females transmitted LACV by bite to suckling mice. Presence of viral plaques in brain material was confirmed for 47 of these mice. Two pups were not tested due to cannibalism by their mothers; these were assumed to be positive because this occured during the period when other pups were exhibiting morbidity. No evidence of infectious virus was observed in 104 of the remaining 115 suckling mice that survived the experiments. Most of the mothers produced a second litter near the end of the test, and three began cannibalizing their first litter; therefore, 11 pups were not available to be tested for the presence of LACV in brain material. They were assumed to be negative

because no morbidity or mortality was observed for >1 week prior to their loss. No nursing mothers seroconverted to LACV, indicating that virus could not have been passed during suckling.

To identify QTL influencing the ability to orally transmit LACV, the data were separated by feeding group and analyzed by IM for a binary trait. The results for FG1 and FG2 are given in Figure 2. No genome locations exceeded the experimentwise 95% threshold LR for declaring a QTL, although three genome regions with elevated LRs are evident. However, the stringency of the experimentwise threshold, while reducing the likelihood of declaring false positive QTL (type I error), also increases the potential for rejecting true positive QTL (type II error; CHURCHILL and DOERGE 1994). In addition, the power of our OT analyses was negatively impacted by the need to evaluate the two feeding groups independently, as this greatly reduced our sample sizes. Therefore, with the small cell values for most markers, individual markers within feeding groups were also analyzed for an association with OT by Fisher's exact test (Table 2). We considered markers with significant associations with OT as identifying tentative QTL. These analyses identified four tentative QTL regions. Of note, the parental alleles at each significant marker that contributed to an increased likelihood of OT did not always

TABLE 2

Associations of marker alleles with oral transmission of La Crosse virus

Marker	LG	Feeding group 1				Feeding group 2			
		P	A	H	B	P	A	H	B
LF179	I	0.0238	2/1[a]	30/2	20/9	0.5218	—	—	—
AtL07	I	0.0563	11/2	26/2	16/8	0.8546	—	—	—
AtL14	I	0.0983	1/1	20/1	6/1	0.0659	1/1	17/11	8/0
LF99	III	0.0226	0/1	19/8	10/0	0.7836	—	—	—
LF296	III	0.0604	3/3	42/8	8/0	0.0504	3/5	47/31	10/1
AtL72	III	0.0965	4/3	37/9	8/0	0.0645	5/7	43/28	9/1
LF334	III	0.0950	7/4	35/7	9/0	0.0299	7/12	40/16	9/5
CRALBP	III	0.0235	2/2	24/7	25/1	0.0175	4/10	28/18	25/9
PABP	Unlinked	1.0000	—	—	—	0.0236	11/5	41/18	7/13

A, *O. hendersoni* homozygote; H, heterozygote; B, *O. triseriatus* homozygote.
[a] Number of individuals not transmitting/number of individuals transmitting for each genotype.

come from the *O. triseriatus* parent. With LGI, one marker (LF179) showed a significant association ($P <$ 0.05) with OT and two markers (AtL07 and AtL14) were suggestive ($P <$ 0.1) for FG1. With FG2, only AtL14 was suggestive of an association with OT. An increased likelihood of OT with LGI markers was associated with *O. triseriatus* alleles. LGIII likely contains at least two QTL associated with each end of the linkage group. At one end, LF99 was significant and LF296 and AtL72 were suggestive with FG1, while LF296 and AtL72 were suggestive with FG2. At the other end, *CRALBP* was significant and LF334 was suggestive with FG1, while both markers were significant with FG2. For both genome regions on LGIII, an increased likelihood of OT with LGI markers was associated with *O. hendersoni* alleles. Finally, a significant association was observed with an unlinked marker (*PABP*), but only with FG2; here the increased likelihood of OT was associated with *O. triseriatus* alleles.

Quantitative genetics of body size: The distribution of wing lengths as a proxy for body size was determined for 164 F$_2$ female progeny (Figure 3). The mean wing length was 3.49 ± 0.22 mm with a range from 2.85 to 3.95 mm. No significant differences in wing length were observed between FG1 (mean, 3.48 mm) and FG2 (mean, 3.50). Therefore, data for both groups were pooled for QTL analyses. As previously indicated (see MATERIALS AND METHODS), wing lengths of the parental species and their F$_1$ hybrids were not obtained.

LR estimates by composite interval mapping exceeded the 95% experimentwise threshold for most marker loci on LGI and one region on LGIII (Figure 4). QTL positions were best defined by the AtL14-LF159 interval on LGI and the AtL72-ATE001 interval on LGIII. Single-marker analyses by linear regression indicated that all markers on LGI and several markers representing both ends of LGIII were significant or suggestive for an association with body size, as well as one marker (AEGI8) on LGII (Table 3). The genome regions on

LGI and LGIII showing significant associations with body size are, therefore, nearly identical to those identified for OT (Table 2). An association between body size and OT was further evidenced when we characterized individuals as above or below the mean body size and performed a comparison with OT using Fisher's exact test (Figure 5). Mosquitoes smaller than the mean were more likely to transmit than were those larger than the mean; observed results were highly significant with FG1 ($P <$ 0.001) and suggestive with FG2 ($P <$ 0.1). These results are contrary to those expected as the smaller body size phenotype is characteristic of the *O. hendersoni* parent.

DISCUSSION

Arbovirus transmission by mosquitoes remains a significant global threat to human health (GUBLER 2002). In this study, we examined the genetic basis for the ability to orally transmit LACV in hybrids of *O. hendersoni* (an inefficient vector) and *O. triseriatus* (the natural vector). Previous studies had shown a relationship between vector competence and vector size (GRIMSTAD and HARAMIS 1984; GRIMSTAD and WALKER 1991; PAULSON and HAWLEY 1991). These studies, however, examined only differences due to environmental effect, *i.e.*, larval nutrition. Here, we identified genetic determinants of both ability to transmit a pathogen orally and adult female body size and then examined the relationship between the two phenotypes. Previous studies (BOSIO *et al.* 1998, 2000; GRAHAM *et al.* 2003) examined the genetic determinants for arbovirus development within the invertebrate vector, but not transmission to a vertebrate host.

We identified four genome regions containing tentative QTL determining virus transmission in the *O. hendersoni/O. triseriatus*/LACV system. We define QTL as tentative, on the basis of observed significant single-marker analyses because no genome regions exceeded the ex-
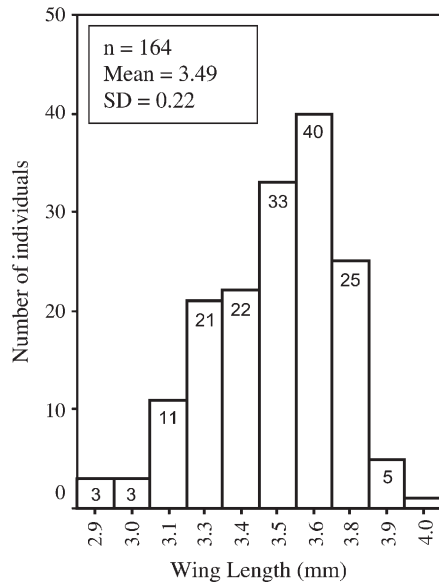
FIGURE 3.—Distribution of wing lengths among 164 females tested for LACV transmission. Values on the *x*-axis represent the midpoint of the interval.

results from these groups were generally consistent, as significant marker associations with LACV transmission were observed for genome regions on LGI (LF179, AtL07, AtL14) and two independent regions on LGIII (LF99, LF296, AtL72 and LF334, *CRALBP*). A fourth genome region, defined by a single marker showing no linkage associations (*PABP*) was associated with LACV transmission, but only with FG2. Of interest, the effect of these tentative QTL on OT did not mirror the parental phenotypes. That is, with two of the four QTL, an increase in OT was associated with alleles from the refractory vector, *O. hendersoni*. Such results are not unusual with studies of complex traits (TANKSLEY and MCCOUCH 1997) and likely reflect epistatic interactions between genes that were uncovered by our interspecific crosses. Although we did not directly determine phenotypes for the parental stocks used to prepare our segregating populations, the dissemination rate has previously been shown to be significantly higher for *O. hendersoni* than for *O. triseriatus* (GRIMSTAD *et al.* 1985). Therefore, one explanation for our results is that the increase in OT due to *O. hendersoni* alleles reflects the effects of genes determining a midgut escape barrier.

Our results indicate that at least three QTL influence body size. Composite interval mapping identified two QTL for body size defined by the AtL14-LF159 interval on LGI and the AtL72-ATE001 interval on LGIII. Single-marker analysis identified a third tentative QTL associated with markers LF334 and *CRALBP* on the opposite

perimentwise 95% threshold for declaring a QTL using IM for a binary trait. Our justification is that experimentwise thresholds are conservative (CHURCHILL and DOERGE 1994) and the power of our OT analyses was reduced by the unanticipated need to evaluate our segregants as two independent feeding groups. However,
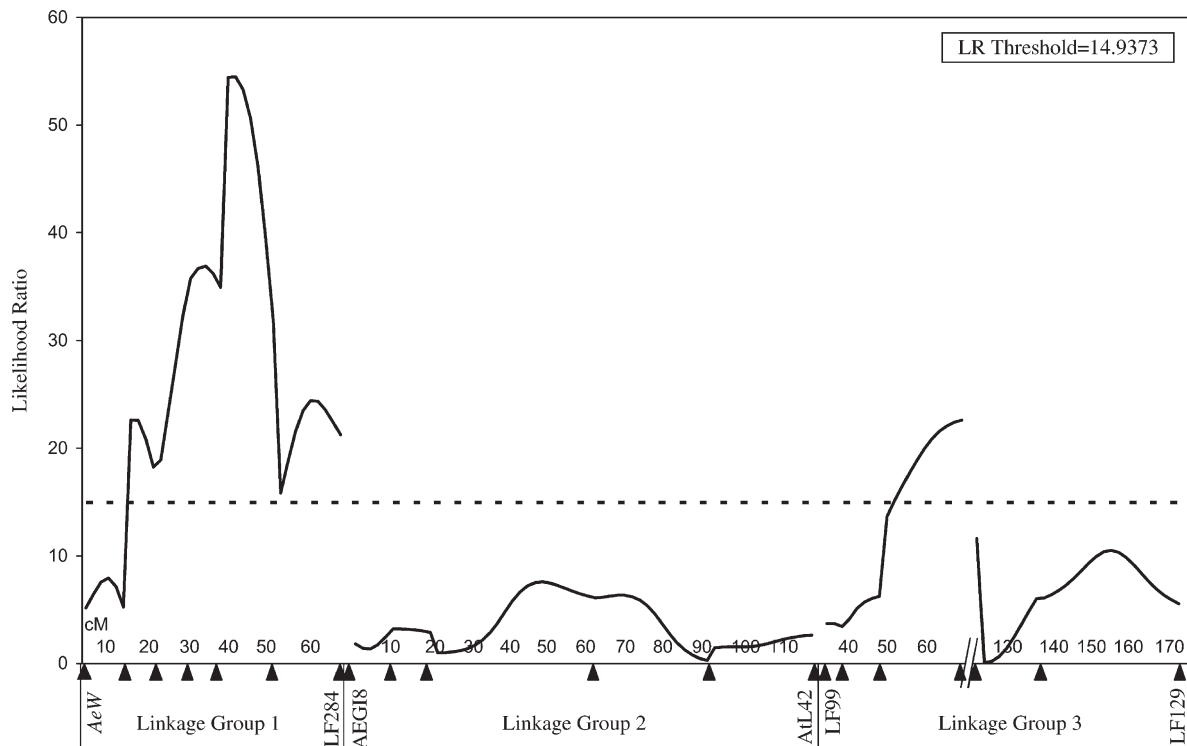

FIGURE 4.—Likelihood ratio profile for identifying quantitative trait loci for body size. The horizontal line represents the experimentwise threshold value for identifying a QTL. Triangles represent the relative location of genetic markers (see Figure 1); two marker names are given for orientation of each chromosome.

Effect of substituting *O. triseriatus* alleles for *O. hendersoni* alleles at genetic markers with a significant association with body size

Marker	LG	*O. triseriatus* Change in wing length (β-coefficient)	95% confidence interval	*P*	Heterozygote Change in wing length (β-coefficient)	95% confidence interval	*P*
AeW	I	—	—	0.1869	0.22	0.15–0.29	<0.0001
LF179	I	0.22	0.10–0.35	0.0004	0.19	0.12–0.25	<0.0001
AtL73	I	0.30	0.14–0.46	0.0003	0.14	0.07–0.21	<0.0001
AtL07	I	0.20	0.12–0.28	<0.0001	0.24	0.18–0.31	<0.0001
AtL14	I	0.20	0.08–0.32	0.0009	0.10	0.03–0.16	0.0024
LF159	I	0.28	0.16–0.40	<0.0001	0.26	0.15–0.36	<0.0001
LF284	I	0.18	0.09–0.27	0.0002	0.25	0.19–0.32	<0.0001
AEGI8	II	0.11	0.02–0.20	0.0163	—	—	0.5523
LF296	III	0.13	−0.02–0.28	0.0831	0.16	0.06–0.26	0.0026
AtL72	III	0.13	−0.01–0.27	0.0607	0.19	0.08–0.29	0.0004
ATE001	III	0.22	0.12–0.31	<0.0001	0.18	0.09–0.27	0.0002
LF334	III	0.12	0.00–0.24	0.0468	—	—	0.1718
CRALBP	III	−0.11	−0.23–0.00	0.0568	−0.09	−0.17 to −0.02	0.0136

end of LGIII. For all loci except *CRALBP*, addition of *O. triseriatus* alleles resulted in larger mosquitoes than did *O. hendersoni* homozygotes.

The genome regions for three QTL determining OT also contain the QTL determining body size. This indicates that these regions contain either single QTL with pleiotropic effects affecting both traits or multiple QTL that independently act on each trait. Of particular note, mosquitoes smaller than the mean were more likely to
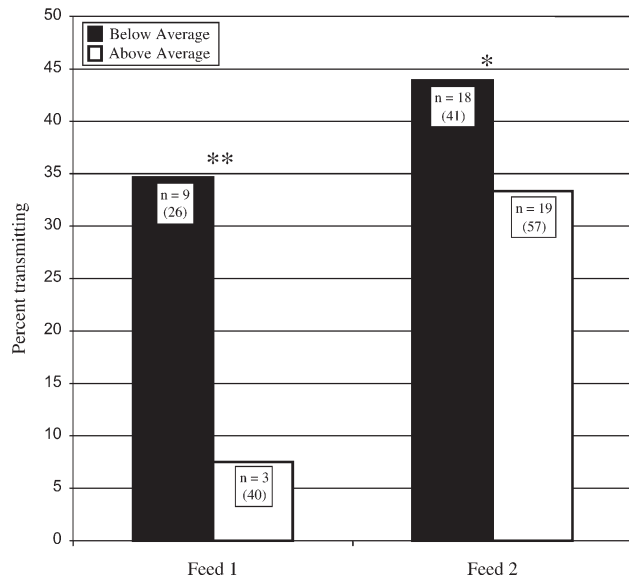

FIGURE 5.—The effect of body size on oral transmission. Bars represent the percentage of individuals below or above the mean body size that transmitted LACV for each feeding group. *n*, total number of individuals that transmitted; parenthetic values are the total number tested in that size class. *$P < 0.1$; **$P < 0.001$.

transmit LACV than were mosquitoes larger than the mean. This was unexpected, as the highly competent vector for LACV transmission, *O. triseriatus*, is larger than the LACV refractory *O. hendersoni* (ZAVORTINK 1972). As previously discussed, this finding undoubtedly reflects the results of epistatic interactions between genes determining OT, wherein for two QTL the increase in OT was associated with alleles from *O. hendersoni*. Interestingly, previous studies had shown that smaller *O. triseriatus* females were more likely to transmit than were their larger counterparts, although the effect in those studies was due to different environmental conditions, namely being starved or well-fed as larvae (GRIMSTAD and HARAMIS 1984; GRIMSTAD and WALKER 1991).

The interspecific linkage map produced from *O. hendersoni* female by *O. triseriatus* male crosses spans 382.5 cM. This is 111.8 cM larger than the intraspecific linkage map generated for *O. triseriatus* (ANDERSON *et al.* 2001). Much of the difference in size is found on LGII, which is ∼2.5 times larger in the interspecific crosses. In addition, LGI is ∼50% larger than the intraspecific linkage map; however, the previously described linear orders of individual marker loci (ANDERSON *et al.* 2001) appear to be conserved in the interspecific map. Linkage group III is essentially the same size in both maps, and large recombinational distances across the likely centromere region (*e.g.*, ATE001 and LF334 in Figure 1) are common to the two maps. Some of the observed increase in LGII size may be attributed to the use of genetic markers lying more distally on the chromosome (*e.g.*, AEGI8) than those in the previously reported map. Lastly, marker *PABP* remained unlinked in this study. This marker is closely linked in *A. aegypti* to another marker (LF377) that was unlinked in the intraspecific

map (ANDERSON *et al.* 2001) and may represent a distal chromosomal arm separated by sufficient recombination as to appear unlinked in both this and our previous study.

Significant deviations from the expected Mendelian ratios were evident for most of the markers. Such distortion may represent the presence of segregation distortion loci (VOGL and XU 2000) or lethal loci (*e.g.*, SEVERSON *et al.* 1995) and may have an impact on identification and localization of QTL. However, distortion of segregation ratios may result in misplacement of the QTL, but not its presence (SCHÄFER-PREGL *et al.* 1996; LU *et al.* 2002). Indeed, segregation distortion in mosquito crosses has been noted in identifying QTL for Plasmodium susceptibility (SEVERSON *et al.* 1995) and for DDT resistance (RANSON *et al.* 2000).

The relative inability of *O. hendersoni* to transmit LACV is not due to an incompatibility, since the virus readily replicates in all tissues, including the salivary glands (GRIMSTAD *et al.* 1985; PAULSON *et al.* 1989, 1992). Thus, there must be some barrier to its escape from the salivary glands, although whether this is physical or physiological is unknown. Nutritional deprivation of *O. triseriatus* larvae leads to a reduction in the thickness of the midgut basal lamina; virus subsequently passes through this potential barrier more easily (GRIMSTAD and WALKER 1991). This precedent for a physical barrier to virus transmission in this system suggests that a similar barrier may be present in the salivary glands of *O. hendersoni*. Three possible mechanisms to the inhibition of viral transmission have been suggested (HARDY *et al.* 1983): (1) sufficient time was not allowed for development of a salivary gland infection before transmission trials were attempted, (2) virus was shed from the salivary glands during a previous sugar meal(s) and had not reaccumulated prior to transmission studies, and (3) a physical barrier similar to the midgut escape barrier existed. This study and that of PAULSON and GRIMSTAD (1989) eliminated the first two as possibilities. Transmission trials were performed after a sufficient period of extrinsic incubation (here 26 or more days) such that salivary glands should have been fully infected and infective. Additionally, the sugar source was removed >24 hr before transmission was attempted, and the salivary glands should have been "restocked" with sufficient virus to infect suckling mice.

Two hypotheses for the inability to transmit virus in the saliva have been put forth following electron microscopic studies of virus-infected salivary glands. JANZEN *et al.* (1970) have shown that chikungunya virus replicates in large numbers in the proximal portions of the lateral salivary gland lobes of *A. aegypti*. However, in these areas, the virus may be too large to enter into the relatively narrow salivary duct openings. Additionally, filamentous areas of the duct could further act as a physical barrier by blocking passage through the channels into the ducts (JANZEN *et al.* 1970). Second, mature virions may accumulate in large masses in areas where there is effectively no means of entering the lumen. Such is the case in *O. triseriatus* salivary glands infected with EEEV (WHITFIELD *et al.* 1971), although large numbers of enveloped viruses were also present in the lumen.

Alternately, there may be physiological differences in the saliva of the two species. Previous studies have shown that mosquito saliva potentiates infections with some viruses, perhaps by inhibiting antiviral innate immune responses (EDWARDS *et al.* 1998; LIMESAND *et al.* 2000, 2003). It is possible that salivary proteins in *O. hendersoni* do not inhibit immune responses to the same level as those of *O. triseriatus*. However, this seems unlikely, as saliva from a diverse group of mosquitoes (*O. triseriatus*, *A. aegypti*, and *Culex pipiens*) were shown to enhance Cache Valley virus infection in mice (EDWARDS *et al.* 1998).

It is currently unknown whether any one of these hypotheses or a different explanation is responsible for the difference in oral transmission seen between *O. hendersoni* and *O. triseriatus*. A single study has examined the salivary glands of *O. triseriatus*, but in relation to infection with EEEV (WHITFIELD *et al.* 1971). Thus there is no literature describing the comparative morphologies of the salivary glands in the two species examined here, nor is there information concerning infection with and replication of LACV on an electron microscopic level in these species. Similarly, no studies have examined the effects of the saliva of these two species on LACV infection, although viremias have been shown to be higher in animals infected by *O. triseriatus* feeding than in those inoculated by syringe (PANTUWATANA *et al.* 1972).

In summary, we have identified four QTL conferring the ability to orally transmit LACV in interspecific hybrids of *O. hendersoni* and *O. triseriatus*. The genome regions associated with three of these QTL also contain QTL controlling the development of body size, suggesting that these regions contain either single genes with pleiotropic effects or multiple linked genes independently determining each trait. This study, together with those of GRAHAM *et al.* (1999, 2003), begins to address the impact that the genetic architecture of *O. triseriatus* and *O. hendersoni* has on the continued transmission of LACV in the United States. Further work is needed to identify and characterize the particular genes responsible for OT in the vector *O. triseriatus* and the nonvector *O. hendersoni*. Identification of such genes could ultimately lead to the identification of novel methods for interruption of the LACV transmission cycle and, potentially, those of other arboviruses.

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