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DISSERTATION

GENETIC ANALYSIS OF TRANSOVARIAL TRANSMISSION OF LA CROSSE VIRUS IN THE EASTERN TREEHOLE MOSQUITO, *Aedes triseriatus*

Submitted by

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Department of Microbiology

In partial fulfillment of the requirements

for the degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall, 1999

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COLORADO STATE UNIVERSITY

November 5, 1999

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY DOUGLAS HUME GRAHAM ENTITLED **"GENETIC ANALYSIS OF TRANSOVARIAL TRANSMISSION OF LA CROSSE VIRUS IN THE EASTERN TREEHOLE MOSQUITO, AEDES TRISERIATUS"** BE ACCEPTED AS FULFILLING IN PART THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT OF DISSERTATION

GENETIC ANALYSIS OF TRANSOVARIAL TRANSMISSION OF LA CROSSE VIRUS IN THE EASTERN TREEHOLE MOSQUITO, *AEDES TRISERIATUS*

The genetic basis of transovarial transmission (TOT) of La Crosse virus in *Aedes triseriatus* was investigated through selection experiments on two mosquito strains. The Holmen strain was subject to selection for TOT refractoriness, the AIDL strain for permissiveness to TOT. Response to selection for a low filial infection (FI) rate was rapid, decreasing from 18% to 3% in three generations. However, no response to selection for permissiveness was observed in the AIDL strain: the average FI rates through four generations fluctuated between 25% and 40%. By contrast, TOT rate in both strains showed a consistent response to selection in both directions. These patterns were consistent with a model in which TOT is controlled by a single genetic locus and permissiveness is conditioned by dominant alleles; while FI rate is not under genetic control, being influenced by environmental factors associated with the mosquito and virus.

Additionally, a RAPD-SSCP linkage map consisting of 72 polymorphic markers was constructed for *Ae. triseriatus*. The estimated size of individual chromosomes was 93 cM for chromosome I, 63 cM for chromosome II, and 37 cM for chromosome III. The estimated length of the entire linkage map was 193 cM, with an overall resolution of one marker every 2.7 cM. This map was used to explore the genetic basis of TOT and

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female-biased sex ratios consistently observed in our *Ae. triseriatus* strains. In quantitative trait locus (QTL) mapping experiments carried out on separate families, TOT and sex were scored as binary quantitative characters. Two QTLs affecting TOT were mapped to chromosome I and four QTLs affecting sex ratio were also located on chromosome I.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

A. Introduction

The world's population today arguably faces as great a public health threat from arthropod-borne disease as it did in the heady days of the 1950s when global eradication of these diseases through the elimination of their vectors, particularly through the use of synthetic insecticides such as DDT, seemed a real possibility. Since that time, a number of ecological and demographic factors have contributed to a dramatic global resurgence in arthropod-borne disease, particularly dengue fever and malaria (Gubler and Clark, 1995; Butler, 1997). Much of this resurgence has been associated with human-induced ecosystem perturbations favoring the proliferation and geographic spread of vector populations. These include dam construction (Gratz, 1999), deforestation, irrigation and other development projects (Epstein, 1995), unchecked population growth and urbanization (Monath, 1994; Gubler, 1996), and global warming (Patz *et al.*, 1995). Add to this a widespread increase in resistance to insecticides (Mouches *et al.*, 1987; Thomson *et al.*, 1993) and the challenges of controlling and eradicating arthropod-borne disease seem daunting indeed.

Over the past 15 years, however, advances in molecular genetic techniques have provided biologists who study arthropod disease vectors with powerful new tools to address these challenges. With the advent of techniques such as genetic cloning, genomic

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mapping, and transformation, genes can be identified and manipulated in ways barely envisioned a generation ago. Not surprisingly, considerable attention is being devoted to the molecular genetic characterization and manipulation of factors influencing vector competence (i.e. the ability to transmit pathogens), particularly in mosquitoes. In a departure from traditional public health approaches, new strategies are being contemplated that are based on population replacement rather than reduction or eradication: replacing wild mosquito populations with genetically modified ones incapable of transmitting pathogens (Collins, 1994; Curtis, 1994; O'Brochta and Atkinson, 1998). To this end, a number of research initiatives are targeted at developing transformation techniques in mosquitoes as well as elucidating the genetic determinants that condition the ability of mosquitoes to transmit pathogenic filarial worms, protozoa, and viruses (Besansky and Collins, 1992; Crampton *et al.*, 1994; Carlson *et al.* 1995).

One major area of research in bioengineering incompetent mosquito vectors is based on the transient expression of exogenous sequences via viral and plasmid expression systems. Anti-viral expression systems based on the alphavirus Sindbis (SIN) have been used with some success in mosquitoes. Double subgenomic SIN (dsSIN) vectors expressing an antisense portion of the La Crosse (LAC) virus RNA were shown to intracellularly immunize certain cells and tissues in *Ae. triseriatus* mosquitoes against subsequent oral infection with LAC virus (Powers *et al.*, 1996). Similarly, dsSIN vectors expressing an antisense portion of Dengue-2 virus in female *Ae. aegypti* effectively prevented salivary gland infection and transmission of this virus (Olson *et al.*, 1996). While capable only of transient cytoplasmic expression, the dsSIN system has provided

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proof-of-principle that pathogen transmission can be blocked in the natural vector through genetic engineering.

The logical next step with experiments of this kind is to integrate the sequences of interest into the mosquito's genome. Given the widely successful use of *P* element-derived vectors in Drosophilids, early efforts to transform mosquitoes were likewise based on *P*-mediated integration. Although these efforts met with only modest success (Miller *et al.*, 1987; McGrane *et al.*, 1988; Morris *et al.*, 1989), stable transformation of *Ae. aegypti* was recently accomplished using the *Mariner* element of *Drosophila* (Coates *et al.*, 1998) and the *Hermes* element of *Musca domestica* (Jasinskiene *et al.*, 1998).

Several research teams have sidestepped the task of directly transforming the arthropod vector genome by focusing instead on exploiting naturally occurring bacterial symbionts as vehicles for introducing exogenous sequences into the arthropod. In theory, the desired sequences can be introduced, expressed, and the products secreted in these simple bacterial systems with greater ease than in complex eukaryotic insect tissues (Beard *et al.*, 1993a). To date, two midgut-associated endosymbionts of medically important vectors have been successfully transformed using plasmid-based constructs: the Rickettsia-like organisms in the tse-tse fly (Beard *et al.*, 1993b) and *Rhodococcus rhodnii* of *Rhodnius prolixus*, the reduviid bug that vectors *Trypanosoma cruzi*, the agent of Chagas disease (Beard *et al.*, 1992). In the case of *R. prolixus*, modulation of vector competence has been achieved (Durvasula *et al.*, 1997).

The notion of using transgenic mosquitoes to control vector-borne disease is politically and ethically controversial and fraught with significant logistical obstacles (Spielman, 1994; Ashburner *et al.*, 1998). Nonetheless, even if transgenic technology

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fails to realize its potential in the field, the ability to routinely transform mosquitoes in the laboratory would be invaluable to efforts to characterize and manipulate genetic factors that condition vector competence.

The work described in this dissertation addresses a component of vector competence: the ability of mosquitoes to transovarially transmit virus to their progeny. Transovarial transmission (TOT) is an important mechanism by which certain arthropod-borne (arbo-) viruses are maintained and amplified in nature. The following chapters describe the mapping of quantitative trait loci (QTLs) which condition TOT of La Crosse (LAC) virus in the eastern treehole mosquito *Aedes triseriatus*. Identification of genes that condition TOT of arboviruses in mosquitoes could provide novel approaches for the control of these pathogens.

This review begins with a summary of knowledge regarding TOT of arboviruses in mosquitoes. The significance of TOT in arboviral epidemiology is discussed, as are the mechanisms by which it occurs and the factors that influence it. Additionally, the *Ae. triseriatus*/LAC virus system is examined in detail as a potential model for studying TOT in other vector/virus relationships. The section concludes with a discussion of the genetic basis of TOT, a key consideration in terms of efforts to map the genes involved, and ultimately to genetically manipulate this trait in the vector. The second section reviews the various markers used in mapping genetic traits, with particular emphasis on mosquitoes. The final section briefly outlines the objectives in each of the following chapters.

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B. TOT and its significance in arboviral epidemiology

1. Introduction

At present, over 500 viruses have been categorized as arboviruses (Karabatsos, 1985). They comprise a heterogeneous group of viruses sharing the biological definition of "viruses maintained in nature principally, or to an important extent, through biological transmission between susceptible vertebrate hosts by haematophagous arthropods; they multiply to produce viremia in the vertebrates, multiply in the tissues of the arthropods, and are passed on to new vertebrates by the bites of arthropods after a period of extrinsic incubation" (World Health Organization, 1967). Many arboviruses are important veterinary and public health pathogens and cause significant morbidity and mortality worldwide. Dengue fever, dengue hemorrhagic fever, yellow fever, Japanese encephalitis, equine (eastern, western, and Venezuelan) encephalitis, Rift Valley fever, Crimean-Congo hemorrhagic fever, and vesicular stomatitis are just a few examples of medically and/or economically important diseases caused by arboviruses. Transmission cycles range from the relatively simple, involving only a single arthropod and vertebrate, to the highly complex, involving numerous vertebrate and arthropod species. Some arboviruses are vectored by ticks, others by sandflies; however, the majority are transmitted by mosquitoes, and the following review focuses primarily on those arboviruses that are mosquito-borne.

One of the enigmas in the epidemiology of many mosquito-borne arboviruses is how the viruses survive during periods of adverse climatic conditions (i.e. winter in temperate regions and the dry season in the tropics) when the vectors are inactive or few in number.

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A number of mechanisms have been postulated to explain how arboviruses are maintained during these periods. These include (a) persistence in the known vertebrate host with delayed, chronic, or recurrent viremia, (b) involvement of unknown accessory hosts or vectors, such as reptiles and amphibians, as maintenance hosts, (c) periodic reintroduction of the virus into an area, for example by migrating birds, (d) persistence in the arthropod vector which survives the adverse conditions through hibernation or diapause, and (e) TOT from an infected vector directly to its offspring (Reeves, 1974; Rosen, 1987; Turrell, 1988).

In support of hypotheses a through d, the data collected thus far have been, in certain cases, provocative, but on the whole, inconclusive. For example, western equine encephalitis (WEE) virus was isolated from white-crowned sparrows collected in December and January (Hardy, 1967). However, subsequent studies indicated these birds probably had current rather than chronic infections, and none of the several thousand mosquitoes that were experimentally allowed to feed on these birds become infected (Hardy, 1967). In similar fashion, the other hypotheses in this group, suffer from a lack of strong supporting data such that no one of them suffices to explain the endemic persistence of arboviruses.

TOT, however, is an especially compelling explanation for virus persistence. Virus is protected within the egg, the stage in the *Aedes* spp. life cycle most resistant to extreme environmental conditions. Also, direct parent to progeny transmission of virus effectively increases the probability of perpetuating the virus life cycle since infected progeny are capable of orally transmitting virus during their first bloodmeal (see explanation below). TOT was first conclusively demonstrated in mosquitoes some 25

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years ago (Watts *et al.*, 1973; Watts *et al.*, 1974; Pantuwantana *et al.*, 1974), and has since been shown to be an established component in many mosquito-borne arbovirus life cycles.

TOT refers to the transmission of an arbovirus from an infected female mosquito to her progeny. Following the ingestion of a viremic blood meal, virus disseminates to the ovaries and infects one or more oocytes through a process which remains only superficially understood (section B3). Once an infected egg is laid, virus propagates in the tissues of the developing embryo. It remains more or less quiescent until the egg hatches, whereupon it reinitiates and/or increases replication and is transmitted through all subsequent metamorphic stages (Chandler *et al.*, 1998). An adult female arising from such an embryo is capable of transmitting virus upon emergence. TOT is confirmed by isolating virus from non-biting mosquito stages (eggs, larvae, pupae, or males) or from adult females prior to their first blood meal.

2. Evidence of TOT of arboviruses in mosquitoes

Laboratory and field studies have shown TOT to be a feature of numerous viruses in the families Bunyaviridae, Togaviridae, Reoviridae, and Rhabdoviridae. Vector species capable of TOT include ticks (*Dermacentor* sp., *Hyalomma* sp., *Rhipicephalus* sp., *Haemaphysalis* sp., *Ornithodoros* sp., *Ixodes* sp.), mosquitoes (*Aedes* sp., *Culex* sp., *Culiseta* sp., *Haemagogus* sp.), and sandflies (*Phlebotomus* sp., *Lutzomyia* sp.)(Tesh, 1984; Turrell, 1988). TOT was first observed in ticks (Hoogstraal, 1966; Burgdorfer and Varma, 1967). In the laboratory, Syverton and Berry (1941) demonstrated "hereditary transmission" of WEE virus in the wood tick *Dermacentor andersoni*. TOT of Crimean-

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Congo hemorrhagic fever virus was shown in *Hyalomma marginatum*, *Rhipicephalus rossicus*, and *Dermacentor marginatus* (Hoogstraal, 1979); subsequently, TOT of Nairobi sheep disease virus was shown in *Rhipicephalus appendiculatus* (Davies and Mwakima, 1982). Vertical transmission of several flaviviruses has also been demonstrated in *Ixodes ricinus* and *I. persulcatus* (Hoogstraal, 1966; Karabatsos, 1985). A number of arboviruses have been shown to be maintained through TOT by sandflies. Among these are the viruses that cause sandfly fever (vectored by *Phlebotomus papatasi*; Tesh and Modi, 1983) and vesicular stomatitis (*Lutzomyia trapidoi*; Tesh *et al.*, 1972).

Vertical transmission in mosquitoes is restricted almost exclusively to viruses in three taxa: the Bunyavirus genus of the family Bunyaviridae, the Flavivirus genus of the family Flaviviridae, and the Alphavirus genus of the family Togaviridae. Vertical transmission, as opposed to the specific process of TOT, is defined as parent to progeny transmission of virus by any mechanism. TOT was first demonstrated in field and laboratory studies with LAC virus and its natural vector *Aedes triseriatus* (Watts *et al.*, 1973, Pantuwatana *et al.*, 1974; section B5 below). These experiments heralded what can be considered the heyday of arboviral TOT research in mosquitoes, a period lasting well into the mid 1980s during which advances in viral antigen and nucleic acid detection techniques enabled the demonstration of TOT in numerous mosquito/virus systems. Following the pioneering work with LAC virus, a number of closely related bunyaviruses were shown to be transovarially transmitted: snow shoe hare virus (transmitted by *Ae. communis*; McLean *et al.*, 1975), Keystone virus (*Ae. atlanticus*; Le Duc *et al.*, 1975), Tahyna virus (*Cs. annulata*; Bardos *et al.*, 1976), Trivittatus virus (*Ae. trivittatus;* Andrews *et al.*, 1977), Jamestown Canyon virus (*Ae. triseriatus*; Berry *et al.*, 1977),

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California encephalitis virus (*Ae. dorsalis*; Crane and Elbel, 1977; Turrell *et al.*, 1980), and San Angelo virus (*Ae. albopictus*; Tesh and Schroyer, 1980; Tesh, 1980).

Vertical transmission of flaviviruses has been documented in numerous mosquito species; however, the parent to progeny transmission in these cases is not transovarial *per se*, but transovum. During transovum transmission, infection occurs late in development as eggs pass through the oviducts and calyx during oviposition. If these structures contain large amounts of virus, infection of the egg can occur since the chorion and micropyle apparatus are still permeable at this stage (Clements, 1992). The vertical transmission of yellow fever virus in *Ae. aegypti* was shown to occur by transovum transmission (Rosen *et al.*, 1989), and it is widely assumed that vertical transmission of all flaviviruses occurs by this mechanism.

Examples of flaviviruses vertically transmitted in mosquitoes include: yellow fever virus (transmitted by *Ae. aegypti*; Aitken *et al.*, 1979; Tesh *et al.*, 1979; Beaty *et al.*, 1980; Fontenille *et al.*, 1997; Thonnon *et al.*, 1998), Japanese encephalitis virus (*Cx. tritaeniorhynchus* and *Ae. albopictus*; Rosen *et al.*, 1978, 1980; Tesh *et al.*, 1979), all four serotypes of Dengue virus (*Ae. aegypti* and *Ae. albopictus*; Jousset, 1981; Rosen *et al.*, 1983), Murray Valley virus (*Ae. aegypti*; Kay and Carley, 1980), Kunjin virus (*Ae. albopictus*; Tesh, 1980), and St. Louis encephalitis virus (*Cx. pipiens*, *Cx. tarsalis, and Cx. Quinquefasciatus*; Francy *et al.*, 1981; Hardy *et al.*, 1980, 1983). Additional examples involving more obscure flaviviruses are reviewed by Tesh (1980, 1981).

In contrast to the bunyaviruses and flaviviruses, evidence of vertical transmission (either by TOT or transovum) of alphaviruses in mosquitoes has been far more elusive. This has led some researchers to conclude that alphaviruses in temperate regions are

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maintained transseasonally by a different mechanism. Eastern equine encephalomyelitis virus was isolated once from pooled males and larvae of *Cs. melanura* (Chamberlain and Sudia, 1961). TOT was also demonstrated in *Ae. vigilax* experimentally infected with Ross River virus (Kay, 1982). This virus was also isolated from pools of *Ae. vigilax* and *Ae. tremulus* males caught in Western Australia (Lindsay *et al.*, 1993). Western equine encephalomyelitis virus was isolated from adults collected as larvae in coastal California (Fulhorst *et al.*, 1994).

Interestingly, the filial infection (FI) rate, the proportion of an infected female's progeny that is infected, differs among various taxa. The average FI rates obtained with bunyaviruses have generally ranged between 3% and 50% (Turrell *et al.*, 1982; Tesh, 1980, Miller *et al.*, 1982), with some being as high as 71% (Miller *et al.*, 1977). In contrast, FI rates obtained for flaviviruses and alphaviruses have rarely exceeded 1% (Tesh, 1984). The reason for this difference and its significance are unknown.

3. The mechanism of TOT of arboviruses in mosquitoes

For TOT of an arbovirus to occur in a mosquito, the egg must become infected at some stage in its development from a germinal cell, and the infection must persist through oocyte enlargement until the chorion is formed and the egg is fertilized. In theory, this can occur in one of three ways: a) An uninfected female ingests a viremic blood meal. After replication in the midgut epithelium, virus disseminates through the hemolymph to the ovaries and infects one or more oocytes. b) An uninfected female mates with a transovarially infected male and becomes infected through venereal transmission during mating. This can lead to a disseminated infection as described above

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or, in theory, direct non-transovarial male to progeny infection. c) A female mosquito harbors a stabilized infection of her germinal cells and virus is maintained over the generations by maternal inheritance (Leake, 1984). The evidence for each of these mechanisms is considered below.

a. TOT resulting from oral infection. When a female mosquito feeds, the blood meal fills the midgut in a posterior to anterior direction. Thus, following a viremic blood meal, epithelial cells lining the posterior midgut are the first to become infected; virus later reaches the foregut tissue either by contact with blood or cell to cell spread. Virus then undergoes several cycles of replication within the midgut epithelium before escaping into the hemocoel where it circulates in the hemolymph, gaining access to other organs and establishing a disseminated infection (Leake, 1984). Some researchers have detected disseminated infections with little or no evidence of midgut infection. This phenomenon, known as a "leaky midgut", results when virus passes through intercellular spaces in the midgut without first infecting and replicating within the midgut epithelial cells (Hardy, 1988). Infection of the various organ systems is assumed to occur more or less randomly as virus circulates in the hemolymph. Fat body and neural tissue frequently contain the most virus (Leake, 1984); other infected organs may include the heart, pericardial cells, dorsal and ventral diverticula, Malpighian tubules, and hindgut (Beaty and Thompson, 1978). Salivary glands and ovaries tend to become infected later in the course of infection (Leake, 1984).

The time it takes for ingested virus to reach the ovaries and infect the oocytes is of particular interest when considering the role of TOT in the epidemiology of arboviruses. If virus in the first blood meal can disseminate rapidly enough to infect the oocytes in the

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first ovarian cycle, infected progeny will arise from not only this, but all subsequent ovarian cycles. In nature, such a mosquito would need only to survive to her first oviposition in order to transmit virus transovarially.

In published studies, virus was detected in the ovaries between 2 and 24 days post ingestion (Table 1.1). Because oogenesis is initiated soon after the ingestion of a blood meal, and because the formation of the chorion, which is impermeable to virus, occurs relatively early in this process, it is generally thought to be unlikely that virus has sufficient time to escape the midgut, reach the ovaries, and infect the developing oocyte(s) of the concurrent ovarian cycle (Turrell, 1988). For example, Miller *et al.* (1979) observed no TOT of LAC virus to *Ae. triseriatus* progeny from the first ovarian cycle, whereas infection rates among progeny of the second and third ovarian cycles were 43% and 58% respectively. Similarly, in his work with San Angelo virus and *Ae. albopictus*, Tesh (1980) detected no TOT to progeny from the first ovarian cycle, whereas subsequent cycles yielded infected offspring.

Table 1.1 Rate of dissemination of ingested virus to ovaries of infected mosquitoes[a]

Species	Virus	Time until virus was detected in ovaries	Reference
Culex pipiens	JE	14 - 21 days	LaMotte (1960)
Culex tarsalis	WEE	4 - 10 days	Thomas (1963)
Culex pipiens pallens	JE	24 days	Doi (1970)
Culex tritaeniorhynchus	JE	9 days	Doi *et al.* (1967)
Aedes triseriatus	LAC	7 days	Beaty and Thompson (1978)
Aedes triseriatus	LAC	2 days	Chandler *et al.* (1998)
Aedes albopictus	DEN-2	6 days	Kuberski (1979)

[a] Adapted from Leake (1984)

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In contrast, several researchers have detected virus in progeny from the first ovarian cycle. Christensen *et al.* (1978) reported infected progeny from first ovarian cycle eggs of *Ae. trivittatus* that had ingested trivittatus virus. Similarly, Watts *et al.* (1973) reported the detection of LAC virus in larvae from each of four ovarian cycles of *Ae. triseriatus*, although they did not detect virus in eggs from the first two cycles. There are additional reports of infection of the first ovarian cycle, although these involved intrathoracic inoculation of the mosquitoes 5 to 7 days before blood feeding. These include work with yellow fever virus (Aitken *et al.*, 1979; Beaty *et al.*, 1980) California encephalitis (Turrell *et al.*, 1980), and with San Angelo and Kunjin viruses (Tesh, 1980; Tesh and Shroyer, 1980).

The precise mechanism by which virus disseminates to developing oocytes is still only superficially understood. The prevailing view subscribes to a stepwise process of midgut escape, dissemination, and ovarian infection as generally described in the preceding paragraphs. However, Chandler *et al.* (1998; Table 1.1) reported the detection of LAC virus in the calyx of the ovaries of *Ae. triseriatus* only 2 days post-ingestion and 4 days before dissemination from the midgut. This suggests the existence of an alternative mechanism of ovarian infection, perhaps by way of the tracheal system.

The mechanism by which virus infects the oocytes is also poorly understood. The epithelium surrounding each oocyte acts as a mechanical filter and is freely permeable to materials less than 11 nm in diameter (Anderson and Spielman, 1971), yet the diameter of most arboviruses is between 5 and 10 times this figure (White and Fenner, 1994). This suggests that virus must traverse the follicular epithelium by way of infection in order to gain access to the oocyte. During oogenesis, vitellogenin is incorporated into the oocyte

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by way of receptor-mediated endocytosis through clathrin-coated pits on the oocyte's surface (Clements, 1992). It is conceivable that virus can exploit this same mechanism to gain access to the cytoplasm of the oocyte.

b. TOT resulting from male to female venereal transmission. Transovarially infected male mosquitoes have been shown to contain large amounts of virus in their accessory glands and seminal fluid (Beaty and Thompson, 1976). During mating, these males can venereally inoculate the reproductive tract of uninfected females. In theory, virus may then infect the oocytes either directly (non-transovarially) during fertilization or indirectly after first establishing a disseminated infection.

Venereal transmission of an arbovirus in mosquitoes was first demonstrated by Thompson and Beaty (1977; 1978). In these experiments, some venereally infected females developed disseminated infections and were subsequently capable of oral transmission. However, TOT was not assayed in these females as their eggs were not analyzed for virus. Tesh (1980) made similar observations with Kunjin virus in *Ae. albopictus* mosquitoes although, again, filial infection was not measured. Using the LAC virus/*Ae. triseriatus* system, Thompson (1979) did assay TOT to progeny of venereally infected females. Interestingly, TOT was observed only in the second and later ovarian cycles, suggesting that eggs were infected after a disseminated infection in the female and not by direct infection of the eggs during fertilization.

It remains to be seen how important a role venereal transmission plays in the natural maintenance of arboviruses. Beaty and Thompson (1975) noted that TOT of LAC virus occurs with equal frequency to male and female progeny of *Ae. triseriatus*. Conceivably,

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given a large enough pool of infected males, venereal transmission could provide a potentially significant supplement to TOT occurring through oral infection.

c. ***TOT resulting from stabilized infection.*** A stabilized infection of a female mosquito is established when most or all of the germinal cells (oogonia) are infected irrespective of their developmental stage, and virus is transmitted to all of her offspring, and potentially to some of her offspring's offspring. Thus, virus is maintained from one generation to the next solely through maternal (cytoplasmic) inheritance, regardless of the infection status of the male parent (Leake, 1984).

Perhaps the most thoroughly studied model of maternal transmission is that of sigma virus in *Drosophila melanogaster*. Sigma virus occurs naturally in *Drosophila* populations worldwide. Flies experimentally infected with the virus suffer fatal paralysis when exposed to CO_2 gas, whereas uninfected flies become only temporarily anesthetized (L'Heritier, 1970; Seecof, 1968). Occasionally female flies become infected and transmit virus to almost all of their progeny. Further inspection of these females reveals virus not only within the oocytes, but throughout the oogonia (L'Heritier, 1970; Seecof, 1968).

There have been a handful of studies of mosquito-borne arboviruses that report infection rates comparable to those obtained with sigma virus in *Drosophila*. Using San Angelo virus, Tesh and Shroyer (1980) established a chronically infected line of *Ae. albopictus* that had consistent TOT rates of 80% over several generations. TOT rate is defined as the proportion of females that transovarially transmit, regardless of FI rate. Turrell *et al.* (1982) achieved rates of over 90% in a line of *Ae. dorsalis* infected with California encephalitis virus. Miller *et al.* (1977) reported comparable results with *Ae. triseriatus* and LAC virus (section B6). Although not an arbovirus, Matsu virus is

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reported to be maternally transmitted in *Culex quinquefasciatus* in much the same manner as sigma virus in *Drosophila* (Vazeille-Falcoz *et al.*, 1992).

With regard to the mechanisms by which arboviruses are maintained transseasonally, the potential role of stabilized infections merits serious consideration. On the assumption that the generally low (as a whole) FI rates thus far observed with arboviruses (section B2) are representative of TOT rates in nature, a commonly held view is that TOT alone is not sufficient to maintain arboviruses for extended periods without repeated horizontal amplification in vertebrate hosts (e.g. Turrell, 1988; Miller *et al.*, 1977). This view is corroborated by mathematical models of TOT (Fine, 1975; Fine and LeDuc, 1978) based on FI rates observed in laboratory studies.

However, experimental TOT rates may not accurately reflect those occurring in the field is open to question. Tesh (1984) postulated that the low FI rates obtained in these studies may be the result of "non-stabilized" infections, during which infection sets in at the oocyte stage of development (i.e. the conventional model of oocyte infection outlined above). In such cases, virus enters some, but not all the oocytes. A female arising from an infected oocyte produces virus which in turn invades some, but not all of her progeny. This is fundamentally different from an infection involving primordial germ tissue, where infection occurs at the origin of development and ultimately gives rise to far greater numbers of infected mosquitoes.

Although cytoplasmic inheritance occurs in nature, it is difficult to induce experimentally. While it is relatively easy to infect somatic cells with a symbiont or pathogen, establishing a germ line infection is much more difficult (Shroyer and Rosen, 1983). Moreover, the mechanism by which virus infects germ cells, as with oocytes, is

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unknown. Assuming that other arboviruses behave like sigma, San Angelo, California encephalitis, and LAC virus, it is entirely understandable why experimental studies of TOT would yield low FI rates: most of these experimentally infected females probably represented the "non-stabilized" condition. On the other hand, transovarially infected females in nature probably sustain stabilized infections and produce FI rates on the order of 100%. Tesh (1984) concludes this line of reasoning by stating:

> "If this hypothesis is correct, then there must be a subpopulation of germ cell-infected females within any given vector population. This subpopulation would maintain the virus by transmitting the agent from generation to generation to almost all of its progeny, even though the virus infection rate in the total vector population would remain quite low. In this situation, there would actually be two distinct populations—one infected and one not. The ratio of these two populations might be changed from time to time by such things a venereal transmission or infection of new females by feeding on viremic vertebrates, *but the basic mechanism of virus maintenance would be by TOT* [italics added]."

4. Factors affecting TOT of arboviruses in mosquitoes

TOT can be affected by a series of potential barriers beginning with midgut and ending with the follicular epithelium (Figure 1.1). The ability to transmit virus transovarially is inextricably linked to vector competence insofar as the midgut is the first structure encountered by virus in both processes (assuming the mosquito is not already transovarially infected). Thus, some of the same mechanisms that render a mosquito incapable of vectoring a virus (i.e. barriers to midgut infection and/or escape) will have the same inhibitory effect on TOT. Experiments aimed at elucidating the barriers to vector competence for certain arboviruses have shown the midgut to play a predominant role, such as was demonstrated with WEE in *Culex tarsalis* (Hardy *et al.*, 1978).

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Presumably, in certain mosquitoes anatomical barriers to TOT may exist beyond the midgut (Figure 1.1), although these have not yet been discovered. Additionally, there may exist physiologic and molecular barriers to TOT. For example, factors within the hemolymph or oocyte that may have an antagonistic effect on viral replication. This too has not been explored. TOT has also been shown to be influenced by genetic variation in the mosquito and in the virus (section B7). Finally, TOT can be affected by environmental conditions. For example, Hardy *et al.* (1980, 1983) demonstrated that the temperature at which mosquito larvae are reared can influence the ultimate infection rate in the adult progeny: higher temperatures generally gave rise to higher infection rates.

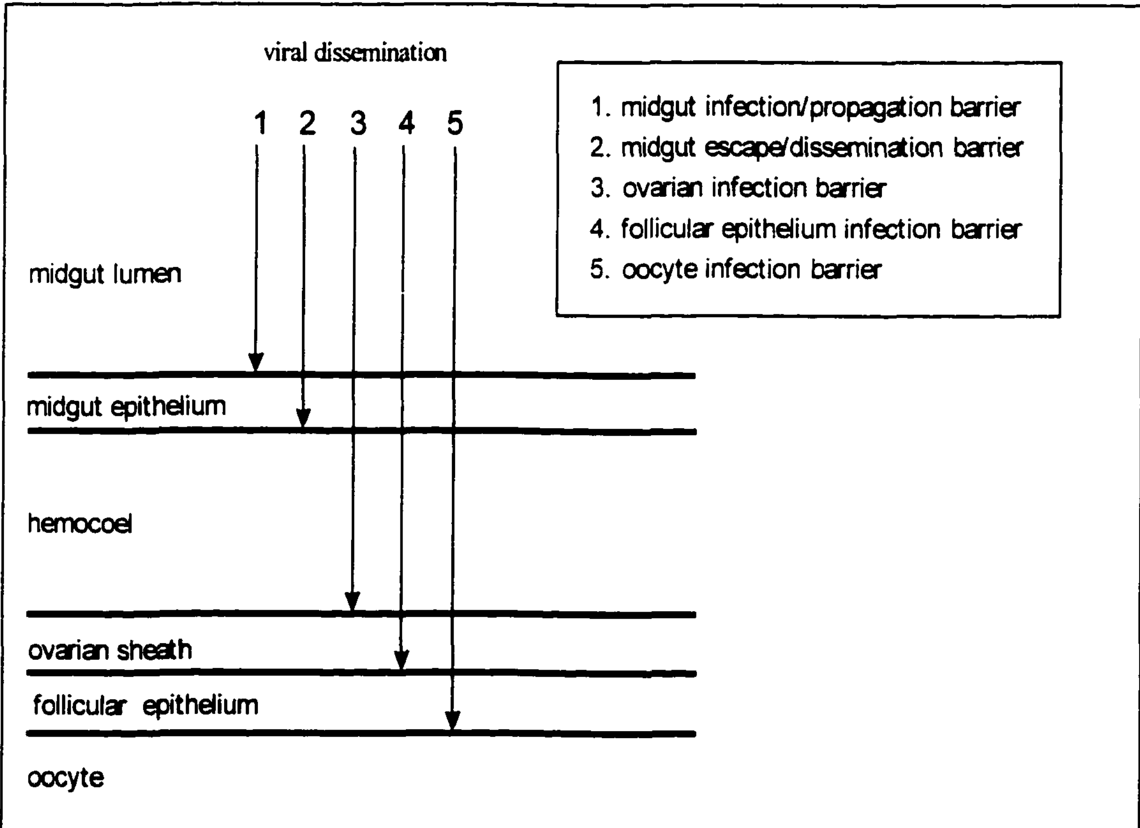

Figure 1.1. Schematic representation of potential barriers to TOT. Horizontal lines represent anatomical barriers to viral dissemination in a mosquito following ingestion of an infectious bloodmeal.

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5. The natural history of La Crosse virus

a. Molecular attributes. LAC virus is a member of the California serogroup, genus *Bunyavirus*, family *Bunyaviridae*. Genomes of the family *Bunyaviridae* contain three segments (large, L; medium, M; and small, S) of single-stranded, negative sense RNA (Schmaljohn, 1996). Virions are spherical, 90 to 100 nm in diameter, and contain four structural proteins: two external glycoproteins (G1 and G2), a nucleocapsid protein (N), and a large protein (L) which is presumed to be a polymerase. Additionally, non-structural proteins are encoded by the M and S segments (Schmaljohn, 1996).

Due to the segmented nature of their genomes, members of the family *Bunyaviridae* can undergo segment reassortment during mixed infections (Pringle, 1996).. Indeed, the great number and diversity of viruses in this family (over 350 members) is thought to be due at least in part to their capacity to evolve by reassortment (Pringle, 1996). Available evidence indicates that reassortment occurs only between viruses in the same serogroup. For example, Chandler *et al.* (1990) demonstrated reassortment between LAC and Snow Shoe Hare virus in orally infected *Ae. triseriatus* mosquitoes. Newly evolved genotypes were transmitted by these mosquitoes both orally and transovarially. Naturally occurring reassortants of LAC have been detected by genotype analyses of field isolates (Klimas *et al.*, 1981).

An interesting property observed in members of the genus *Bunyavirus* is the use of host mRNA to prime viral transcription. A methylated cap-dependent endonuclease associated with the polymerase complex cleaves capped 10- to 18-nucleotide segments from the 5' termini of host cell mRNAs (cap scavenging) to prime viral transcription (Kolakofsky and Hacker, 1991). Cap scavenging is particularly noteworthy in light of the

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LAC virus life cycle: this mechanism may be a factor maintaining viral persistence in overwintering *Ae. triseriatus* embryos. Coregulation of host biosynthesis and viral replication during diapause may promote efficient overwintering of LAC virus. LAC virus has been shown to replicate continuously in metabolically active *Ae. triseriatus* ovaries (induced by bloodfeeding), whereas LAC mRNA and replicative-form RNA were greatly reduced or undetectable in metabolically quiescent ovaries (Chandler *et al.*, 1996). Subsequent studies detected LAC virus replication in both metabolically active (embryonating) and dormant (diapausing) *Ae. triseriatus* embryos (Dobie *et al.*, 1997; McGaw *et al.*, 1998). Additionally, heterogeneity in the sequences of host-derived viral mRNA primers was markedly reduced compared with that seen in mammalian cells, and the predominant sequences changed over time in embryonating and diapausing embryos (Dobie *et al.*, 1997). If viral cap scavenging is restricted to abundantly synthesized or stable host sequences, this could serve to moderate deleterious effects of viral replication on the embryo.

b. Transmission cycle in nature. LAC virus is maintained in forested areas in a natural cycle of transmission between its principal vector, *Ae. triseriatus*, and small rodents, predominantly eastern chipmunks (*Tamias striatus*) and gray squirrels (*Sciurus carolinensis*). *Ae. triseriatus* breeds mainly in root cavities of hardwood trees, but can readily breed in discarded tires and other artificial containers in densely populated suburban areas, thus increasing the risk of human infection. As mentioned above, LAC virus is also maintained in nature through TOT and, to an unknown extent, through venereal transmission (Thompson and Beaty, 1977, 1978).

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*c. **Epidemiology and medical importance.*** LAC virus emerged as a significant human pathogen in the 1970s and remains a significant cause of encephalitis and aseptic meningitis in children in the U.S. (CDC, 1987; Calisher, 1994; Tsai, 1991). Post mortem isolation of the virus from the brain of a child infected near La Crosse, Wisconsin (Thompson *et al.*, 1965) led to the recognition of this virus as the causative agent of LAC encephalitis, the primary cause of arboviral encephalitis in the U.S. Over the past 30 years, the number of reported cases of LAC encephalitis has ranged from 42 to 174 cases per year (Calisher, 1994). LAC virus is known to cause encephalitis in children in essentially all states east of or contiguous to the Mississippi River. Historically, the highest incidence of LAC encephalitis has been reported in the Great Lakes region, in a swath extending from Minnesota to western New York (Calisher, 1994). However, recent work by the Centers for Disease Control in West Virginia has revealed more LAC encephalitis cases in this state alone than in all the Great Lakes states combined (Berry, 1998). This is probably not a new occurrence, but instead reflects a previous lack of diagnosing and reporting by physicians in this area. If and when similar public health initiatives are established in other eastern regions, it may be revealed that LAC encephalitis is far more widespread than historically thought.

6. LAC virus as a model for studying TOT

TOT of LAC virus by *Ae. triseriatus* is one of the best characterized TOT systems. The first published report of TOT of an arbovirus in mosquitoes documented the recovery of LAC virus from eggs, larvae, and adults of experimentally infected *Ae. triseriatus* (Watts *et al.*, 1973). TOT of LAC virus was shown to occur under natural conditions

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with the recovery of virus from field-collected *Ae. triseriatus* larvae (Pantuwatana *et al.*, 1974). In an experiment illustrating the considerable role of TOT in the epidemiology of LAC virus, Miller *et al.* (1977) demonstrated that LAC virus can remain infective to warm-blooded hosts after 8 successive transovarial passages in *Ae. triseriatus.* These data indicated that the virus can persist 4 years or longer in the absence of horizontal amplification in vertebrate hosts.

Every year in temperate regions, LAC virus can be isolated from mosquitoes emerging from overwintering (diapausing) eggs; thus, LAC survives the winter by TOT. TOT rates and FI rates can be as high as 80% to 90% (Miller *et al.*, 1977), and LAC virus is capable of overwintering in eggs of *Ae. triseriatus* for up to two years (Beaty and Thompson, 1975). Thus, *Ae. triseriatus* is an epidemiologically significant vector that serves both as a reservoir for and a vector of LAC virus. Exploiting our understanding of the genetic and physiological mechanisms underlying TOT in the LAC/*Ae. triseriatus* system will enable us to more effectively understand and manipulate this component in other important virus-vector relationships.

7. The genetics of TOT

Virtually nothing is known about the genetic basis for TOT in arthropod vectors or in arboviruses. There very likely is a genetic basis for TOT because some vectors are proficient at TOT of particular viral species while others are not (Rosen *et al.*, 1983; Schopen *et al.*, 1991). Furthermore, in the wild, certain populations of a vector species exhibit consistently greater TOT rates than others (Miller *et al.*, 1982; Woodring *et al.*,

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1998). In addition, the strains of *Ae. triseriatus* used in the experiments described in this dissertation responded to artificial selection on TOT (Chapter 2;Graham *et al.*, 1999a).

A genetic basis for TOT in the virus is also inferred from studies showing variation in vertical transmission rates among virus strains. In *Aedes albopictus* mosquitoes, variations in vertical transmission rates have been observed among 5 different Kunjin virus isolates (Tesh and Schroyer, 1980) and between the four serotypes of dengue virus (Rosen *et al.*, 1983). However, it should be noted that because viral transmission rates cannot be assayed outside the vector, the values reported in these studies were inherently affected to an unknown extent by genetic variation in the mosquitoes.

C. Mapping Genetic Traits

1. Linkage mapping in mosquitoes.

a. Early linkage maps. The theory behind linkage mapping has its origins in classical genetics. It was first developed by Thomas Hunt Morgan and his graduate student Alfred Sturtevant in their work with *Drosophila* during the early part of this century (Lynch and Walsh, 1997). The frequency of offspring with a recombinant genotype resulting from a cross serves as a quantitative index of the linear distance between two loci. Most of the markers used during this era were mutants that caused discernable alterations in phenotype (e.g. morphological or physiological abnormalities). These are typically rare in natural populations, often have significantly lower viability than the wild type, and thus need to be isolated and maintained in special stocks before being mapped. Usually, such stocks can survive the genetic load of only a modest number of such markers. Therefore the mapping of even a moderate number of markers

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requires that they be assembled into common lineages through tedious breeding experiments. Despite these limitations, mutant markers have formed the basis of many linkage maps over the years. In mosquitoes, markers based on morphological mutations and insecticide resistance were used to construct the first linkage maps of *Ae. aegypti* (Craig and Hickey, 1967), *Anopheles quadrimaculatus* (French and Kitzmiller, 1964), *An. gambiae* (Mason and Davidson, 1966), and *Culex pipiens* (Iltis *et al.*, 1965).

b. Allozymes. In the 1960s, the discovery of enzyme polymorphisms in *Musca domestica* (Ogita, 1962; Menzel *et al.*, 1963) and *Drosophila melanogaster* (Wright, 1963; Hubby and Lewontin, 1966) heralded the use of allozymes (allelic forms of the same isozyme), a new class of marker based on differences in electrophoretic mobility in starch and agar gels. Mosquito geneticists were quick to employ these new markers in their genetic analyses, and the ensuing decades saw allozyme linkage maps constructed for a number of mosquito species including *Ae. aegypti* (Munstermann and Craig, 1979), *Ae. albopictus* (Tadano, 1989, 1987, 1984), *Ae. triseriatus* (Matthews and Munstermann, 1990), *Ae. togoi* (Tadano, 1986, 1983, 1982), *Ae. scutellaris* (Pashley and Rai, 1983) *Anopheles albimanus* (Narang and Seawright, 1987), and *Cx. pipiens* (Cheng and Hacker, 1977), among others.

c. Restriction Fragment Length Polymorphisms. In the early 1980s, a new paradigm emerged in linkage mapping. It was based on a new cadre of molecular genetic techniques capable of generating and scoring large numbers of variable marker loci. The technique that launched this revolution is restriction fragment length polymorphism (RFLP) analysis (Botstein *et al.* 1980). The use of RFLP analysis has revolutionized

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human genetics over the past 15 years, and enabled the construction of high density linkage maps of numerous eukaryotic species.

The basic steps involved in RFLP analysis include the isolation of DNA from individuals, digestion with restriction enzymes, separation of the fragments on the basis of size by gel electrophoresis, transfer of the DNA to a membrane by Southern blotting, and probing the membrane with a labeled fragment of DNA that hybridizes to specific target sequences. Individual-to-individual variation in the patterns seen is caused by underlying genetic variation in the target DNA sequence (e.g. a base pair substitution creating or abolishing restriction sites) or sequence arrangements (e.g. insertions or deletions of DNA in the interval spanned by neighboring restriction sites).

A linkage map of *Ae. aegypti* was constructed based on 50 RFLP markers (Severson *et al.*, 1993). These same markers were used to identify mosquito genome regions containing genes determining competence to transmit malarial (Severson *et al.*, 1995a) and filiarial worm (Severson *et al.*, 1994; Beerntsen *et al.*, 1995) parasites. RFLP markers were used to develop comparative linkage maps between *Ae. aegypti* and *Ae. albopictus* (Severson *et al.*, 1995b). RFLP markers have also been employed in *An. gambiae*, most notably to investigate the linkage between a putative diphenol oxidase gene and an esterase locus associated with resistance to malarial parasites (Romans *et al.*, 1991).

d. Simple sequence repeats. Depending on the type of probe employed, different classes of marker loci are revealed. Single-copy loci with unique genomic locations are the most widely employed for mapping. These are visualized when single-copy sequences (either anonymous or corresponding to known, cloned genes) are used as the

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probe. In many species, probes have been discovered that reveal complex, hypervariable patterns consisting of dozens of bands. One class of these are the so-called minisatellites, which consist of core sequences of 20 to 50 nucleotides. These arise from uneven crossover and are found dispersed throughout the genome as tandem arrays of variable numbers of repeats (a.k.a. VNTRs, Variable Number Tandem Repeat) of the core sequence (Jeffrey *et al.*, 1985).

Even more ubiquitous are the microsatellites: tandem repeats of very simple sequences such as di- or trinucleotides (e.g. $[GT]_n$, $[CAC]_n$, etc...) resulting from uneven crossover or slippage replication (Tautz, 1989). Microsatellite probes reveal an enormous amount of polymorphism and have been employed to construct linkage maps of numerous eukaryotic organisms. To date, however, microsatellite markers have been mapped in only one mosquito species, *An. gambiae* (Zheng *et al.*, 1993, 1996; Zheng, 1997; Gorman *et al.*, 1997).

e. ***RAPD-PCR.*** RAPD-PCR (Random Amplified Polymorphic DNA - amplified by PCR) is a powerful new mapping technique capable of identifying many polymorphic regions of a genome simultaneously by using short oligonucleotide primers of arbitrary sequence (Williams *et al.*, 1990). Unlike methods based on microsatellites or RFLPs, RAPD-PCR does not require genomic library construction, sequencing, radiolabeling, or the expensive design and assembly of primers.

High density linkage maps based on RAPD-PCR markers have been constructed for a large number of species including forest trees (Byrne *et al.*, 1995; Plomion *et al.*, 1995), several agricultural crop species (Kurata *et al.*, 1994; Kesseli *et al.*, 1994), the zebrafish (Postlethwait *et al.*, 1994), two species of monkeyflower (Bradshaw *et al.*, 1995), the

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honeybee (Hunt and Page, 1995), a parasitic wasp (Antolin *et al.*, 1996), and the silkworm (Promboon *et al.*, 1995). To date, four mosquito species have been mapped using RAPD-PCR markers: *Ae. aegypti* (Antolin *et al.*, 1996), *Ae. albopictus* (Mutebi *et al.*, 1997), and *An. gambiae* (Favia *et al.*, 1994; Dimopoulos *et al.*, 1996)., and *Ae. triseriatus* (Chapter 3; Graham *et al.*, 1999b)

Despite the significant practical and economical advantages to using RAPD-PCR, this technique does have one drawback when used in mapping. Approximately 90% of polymorphisms detected by analysis of RAPD-PCR on agarose gels segregate as band presence or absence. The dominance of band-present alleles hinders phase determination in F_2 or backcross genotypes needed to identify recombination frequencies. Codominant loci, where complete genotypes can be unambiguously determined, are clearly advantageous in linkage mapping.

However, this limitation can be largely overcome by detecting fine-scale variation within RAPD-PCR fragments by Single-Strand Conformation Polymorphism (SSCP) analysis (Orita *et al.*, 1989; Hayashi, 1991). SSCP analysis is based on the principle that electrophoretic mobilities of single-strand DNA molecules in non-denaturing gels depend upon both size and shape of the fragments. Prior to electrophoresis, double-stranded RAPD-PCR fragments are denatured, then cooled to produce several stable conformations as secondary base pairing occurs among nucleotides on single DNA strands. The length, location and number of intra-strand base pairs determines secondary and tertiary structure of a conformation. Point mutations that affect intra-strand interactions may therefore change the shapes of molecules and alter their mobility during electrophoresis. The SSCP technique detects up to 99% of point mutations in DNA

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molecules 100-300 bp in length and 89% of mutations in molecules 300-450 bp in length (Orita *et al.*, 1989; Hayashi, 1991). Ultimately, SSCP increases both the number of loci and the number of codominant polymorphisms seen in RAPD-PCR products.

2. Mapping quantitative traits

Characters whose phenotypic variation is continuous, influenced by the environment, and determined by the segregation of multiple loci are referred to as quantitative traits. The individual loci which collectively condition a quantitative trait are termed quantitative trait loci (QTLs). When combined with QTL analysis, high density linkage mapping allows identification of the number and effect of genes underlying phenotypic characters. Linkage between genetic markers and QTLs can be detected using t-tests based on association with single markers (Soller *et al.*, 1976, Darvasi *et al.*, 1993), or by interval mapping using likelihood ratio tests involving pairs of markers flanking QTLs (Lander & Botstein, 1989; Knapp *et al.*, 1990; Zeng, 1994). Recent approaches combining saturated linkage maps with statistical techniques such as regression and analysis of variance (ANOVA) have improved the efficiency and resolution of QTL mapping (Zeng, 1994; Kruglyak and Lander, 1995).

The procedure for mapping QTLs in an F_1 intercross is outlined in Figure 1.2. In essence, QTL mapping entails a) partitioning the population into different genotypic classes based on genotypes at the marker locus, and b) using correlative statistics to determine whether the individuals of one genotype differ significantly compared with individuals of other genotype(s) with respect to the trait being measured. If the phenotypes of individuals in each marker class vary significantly (the variation being a

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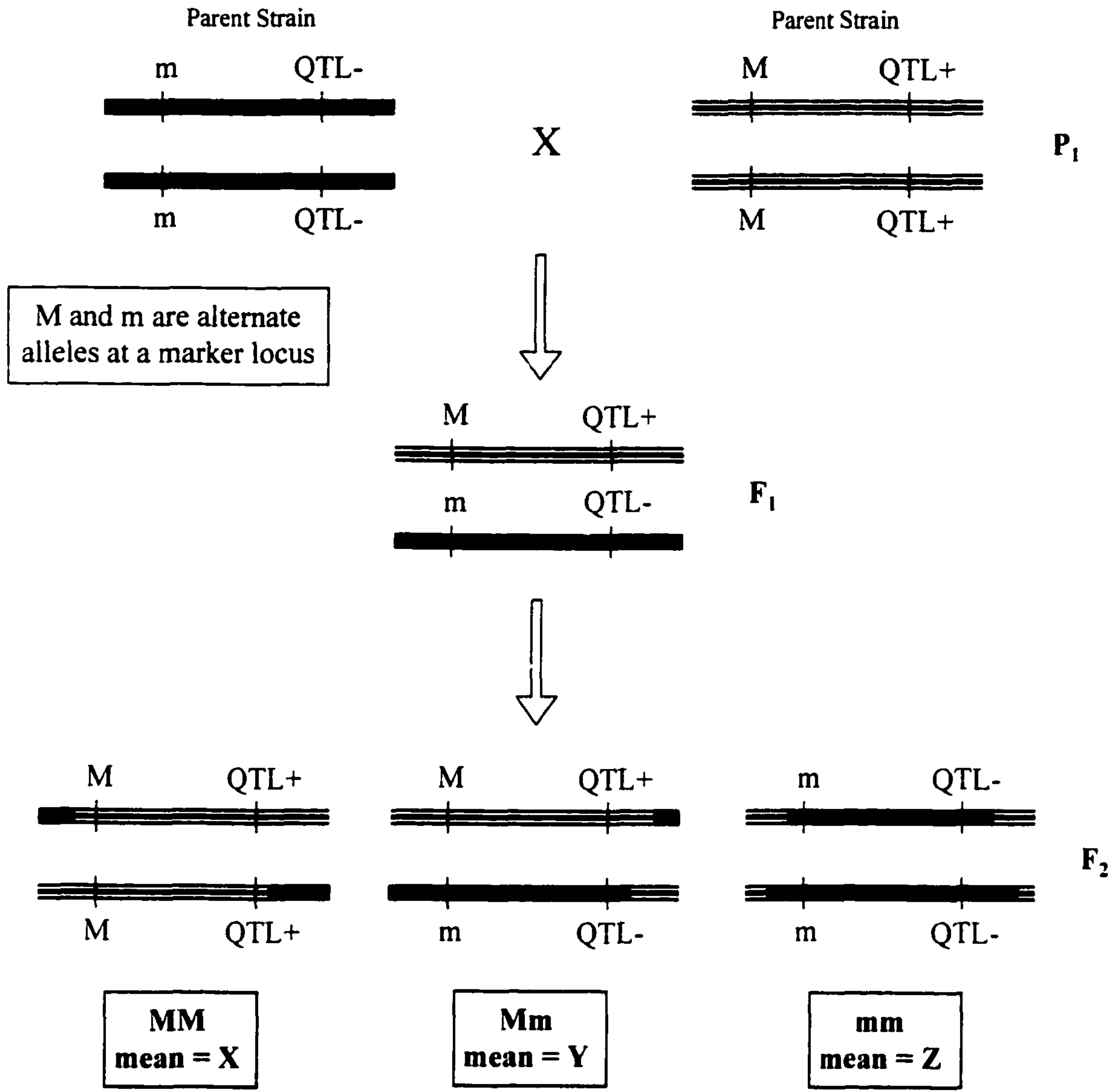

Figure 1.2. Detection of a QTL linked to a marker locus in a theoretical F_2 population. The P_1 generation represents a cross between individuals with extremes in the quantitative trait of interest. The F_1 hybrids are heterozygous at both marker and QTL. The QTL is detected when a statistically significant difference is observed between the means (X, Y, and Z) of F_2 progeny of different marker genotypes (adapted from Tanksley, 1993).

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function of recombinational distance between the marker and the QTL, the additive effect of alleles at the QTL, and the degree of dominance between QTL alleles), it is interpreted that a QTL is linked to the marker locus.

QTL mapping has been employed to analyze a wide range of phenotypes in a number of organisms: reproductive isolation in monkey flowers (Bradshaw *et al.*, 1995), weaver disease in cattle (Georges *et al.*, 1993), cognitive ability (McClearn *et al.*, 1997) and male sexual orientation (Hamer *et al.*, 1993) in humans, foraging and stinging behavior in honeybees (Hunt and Page, 1995; Hunt *et al.*, 1998), and convergent domestication of cereal crops (Paterson *et al.*, 1995), to name just a few.

Several QTL mapping experiments have been carried out in mosquitoes, the focus in each case being on QTLs affecting vector competence. In *Ae. aegypti*, Severson and colleagues (1994) mapped a QTL, recessive with respect to susceptibility to the filarial parasite *Brugia malayi*, on chromosome I, and also identified a second locus of lesser effect on chromosome II. In a similar experiment, two QTLs were identified, one of large effect on chromosome II and another of lesser effect on chromosome III, which condition susceptibility of *Ae. aegypti* to the avian malarial parasite *Plasmodium gallinaceum* (Severson *et al.*, 1995a). Additionally, one major and two minor loci conditioning refractoriness to the simian malarial parasite *P. cynomolgi* were identified in *An. gambiae* (Zheng *et al.*, 1997). Recently, Bosio and colleagues (1999b) identified four QTLs conditioning susceptibility to DEN-2 infection in *Ae. aegypti*.

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D. Research Objectives

The overall aim of this dissertation research was to test the hypothesis that TOT of LAC virus in *Ae. triseriatus* is genetically conditioned in the vector and to map QTLs affecting this trait. Therefore, the specific objectives of this dissertation were to:

1) Subject *Ae. triseriatus* to artificial selection on TOT of LAC virus. The experiments described in chapter 2 test the hypothesis that there exists in *Ae. triseriatus* a genetic basis to the TOT of LAC virus. A common approach to infer that a trait is controlled by genetic, as opposed to environmental, factors is to subject this trait to selection. I selected for refractoriness and permissiveness to TOT of LAC virus in two strains of *Ae. triseriatus*, with FI rate being the phenotype under selection (Graham *et al.*, 1999a).

2. Construct a dense linkage map of the *Ae. triseriatus* genome suitable for mapping QTLs. Chapter 3 describes the construction of the first genetic linkage map for *Ae. triseriatus* using RAPD-SSCP markers. I used this map to test several hypotheses. The first hypothesis is that the recombinational map length of *Ae. triseriatus* based on RAPD-SSCP markers and assembled with current multi-point mapping technology would corroborate earlier measurements based on allozymes and morphological markers (Munstermann, 1989). The second hypothesis is that I should expect the recombinational map length of *Ae. triseriatus* to be considerably larger than that of *Ae. aegypti* and *Ae. albopictus*, the two other mosquito species for which RAPD-SSCP linkage maps have been constructed. This expectation is based on measurements of physical genome size showing the haploid *Ae. triseriatus* genome to contain considerably more DNA than the other two species

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(Black and Rai, 1988). At this stage in my research, I observed widespread female-biased sex ratio among our *Ae. triseriatus* strains. Having just completed the linkage map, I therefore took the opportunity to test a third hypothesis. Namely, that there are QTLs, in addition to the sex locus, that affect female-biased sex ratios in the AIDL strain of *Ae. triseriatus* (Chapter 3; Graham *et al.*, 1999b). If the biased sex ratios I observed were caused by environmental effects, I would expect to see only one QTL associated with the sex locus.

3) Use the linkage map to test the hypothesis that there is a genetic basis to TOT of LAC virus in *Ae. triseriatus* (Chapter 4). If TOT is genetically controlled, I should expect to be able to map one or more QTLs affecting this trait.

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CHAPTER 2

SELECTION OF REFRACTORY AND PERMISSIVE STRAINS OF *AEDES TRISERIATUS* (DIPTERA: CULICIDAE) FOR TRANSOVARIAL TRANSMISSION OF LA CROSSE VIRUS.

A. Introduction

Aedes triseriatus (Say) is the principal vector of La Crosse (LAC) virus, the leading cause of pediatric arboviral encephalitis in the United States (Calisher, 1994). This virus is efficiently transmitted vertically in *Ae. triseriatus*. Rates of transovarial transmission (TOT, the proportion of females transmitting virus to their progeny) and filial infection (FI, the proportion of TOT infected progeny from a given female) can both exceed 70% (Miller *et al.*, 1977). *Ae. triseriatus* and LAC virus share an overlapping distribution comprising most states east of, or contiguous to, the Mississippi River. In northern states *Ae. triseriatus* overwinters by egg diapause, and TOT is the mechanism by which LAC virus survives transseasonally (Beaty and Bishop, 1988).

TOT of LAC virus in *Ae. triseriatus* is one of the best characterized examples of vertical transmission among medically important arthropods (Beaty and Bishop, 1988). Despite this, very little is known of the genetics of TOT. Tesh and Schroyer (1980) demonstrated a genetic component to TOT of San Angelo virus in *Ae. albopictus* (Skuse) and this remains the only published account which explores a genetic basis of TOT. Variation in LAC virus FI rates among different geographic populations and laboratory strains of *Ae. triseriatus* (Miller *et al.*, 1982; Woodring *et al.*, 1998) indicates a possible heritable basis to this trait.

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In the following study I test the hypothesis that there exists a genetic basis in *Ae. triseriatus* for TOT of LAC virus. A common approach for inferring the degree to which a trait is controlled by genetic, as opposed to environmental, factors is to subject the trait to artificial selection. A strong and consistent response to selection is an indication that the trait has a significant genetic basis (Falconer and Mackay, 1996). I selected for refractoriness and permissiveness to TOT of LAC virus in two strains of *Ae. triseriatus*, with FI rate being the phenotype under selection. Elucidating the degree to which this epidemiologically important phenomenon is conditioned genetically in the vector can serve as the basis for future efforts aimed at mapping and characterizing the gene(s) involved.

B. Materials and Methods

***Aedes triseriatus* strains.** I initially attempted to establish *Ae. triseriatus* colonies directly from the field. However, insemination rates were extremely low, even after several generations. I therefore chose to carry out the experiment on fully lab-adapted colonies because efficient mating was critical to the experimental design and my attempts at artificially induced copulation were unsuccessful. The AIDL and Holmen mosquito strains originated from eggs collected at different sites near La Crosse, Wisconsin. The AIDL strain originated in 1983 and has been in colony for ~ 30 generations, whereas the Holmen strain, established in 1992, has been through 15 generations. Initial data on FI rates from the AIDL and Holmen strains were available from previous experiments. These FI rates were sufficiently different between strains (Figure 2.2, P_1 generation) that I continued selecting for a high average FI rate in the AIDL strain, and a low average FI

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rate in the Holmen strain. Each colony was maintained at 20-23°C, 75% relative humidity, and a photocycle of 16:8hr L:D with a 60 min crepuscular period at dawn and dusk. Larvae were fed Tetra-Min® fish food. Adults were housed in 24 x 24 x 24 in flight cages and consisted of 500 – 1,000 individuals per strain.

Virus stock. LAC virus was obtained from the World Health Organization Arbovirus Reference Bank at the Yale Arbovirus Research Unit, New Haven, CT. This virus was originally isolated in 1964 from the brain of a patient with LAC encephalitis (Thompson *et al.*, 1965). The AIDL stock of this virus was passed once in suckling mouse brain and eight times in baby hamster kidney (BHK-21) cells (Chandler, 1995).

Preparation of infectious bloodmeal. LAC virus was grown in BHK-21 cells in Leibovitz L-15 medium containing 10% fetal bovine serum, penicillin, and streptomycin. A cell monolayer (T-75 flask) was incubated with virus at a multiplicity of infection of 0.01 at 37°C for 1 hr., after which supernatant was replaced with fresh growth medium. Viral infection of the monolayer was allowed to proceed until producing moderate to severe cytopathic effects. Supernatant then was harvested by centrifugation at 1000 x g for 10 min., and 5 ml were added to equal parts of a 10% sucrose solution and defibrinated sheep blood (Colorado Serum Co., Denver, CO). Bloodmeal titers ranged between 10^6 and 10^8 Tissue Culture Infectious Dose $(TCID)_{50}$/ml (Karber, 1931) at the beginning of the meal, and decreased by an average of $10^{0.5}$ $TCID_{50}$/ml by the end of blood feeding.

Oral infection of mosquitoes. At 5-7 d post-emergence, female mosquitoes were removed from colony cages and placed into several 1-gallon (3.8 L) containers with mesh fabric lids. They were deprived of sugar and water for 24 and 12 hrs, respectively, before

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blood feeding. The bloodmeal was administered via hanging droplets and artificial membrane feeders (Rutledge *et al.*, 1964). Mosquitoes were allowed to feed for 1 hr. Fully engorged females were returned to their respective colony cages for 2-3 d to maximize the likelihood of mating. Partially engorged females and those that did not feed were discarded (Figure 2.1A)

Collection of eggs. Females were removed from the colony cage and placed individually in 1-pint (500 ml) oviposition containers. Eggs were oviposited on strips of moist paper towel. LAC virus does not transovarially infect progeny from the first ovarian cycle (Miller *et al.*, 1979). Therefore, for each female, I collected one batch of uninfected eggs from the first ovarian cycle, and one that potentially contained infected progeny from the second ovarian cycle. Eggs from the first ovarian cycle (induced from the infectious bloodmeal) were collected and labeled according to mother. The second ovarian cycle was induced by providing a noninfectious bloodmeal consisting, in a 2:1 proportion, of defibrinated sheep blood and 10% sucrose solution. This second bloodmeal was administered separately to each female via hanging droplets. The large number of individual females in pint cartons prohibited the use of artificial membrane feeders. Eggs from the second oviposition were collected and the towel strips were placed in labeled plastic bags (Figure 2.1A)

Assay of viral infection of mosquitoes. Larvae from the second ovarian cycle were reared to the second or third instar, squashed on microscope slides, and assayed for the presence of LAC antigen using anti-LAC antibody/fluorescein isothiocyanate (FITC) conjugate and a fluorescent microscope (Chandler, 1995). Adult females were assayed for disseminated infection by assaying neural tissue in head squashes for viral antigen

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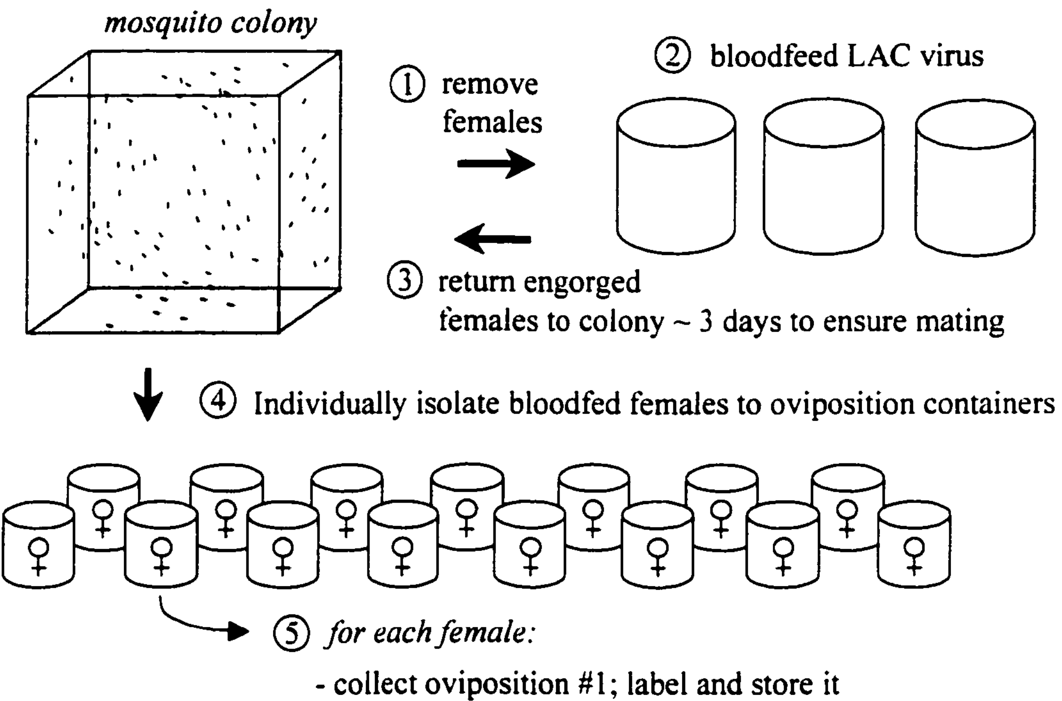

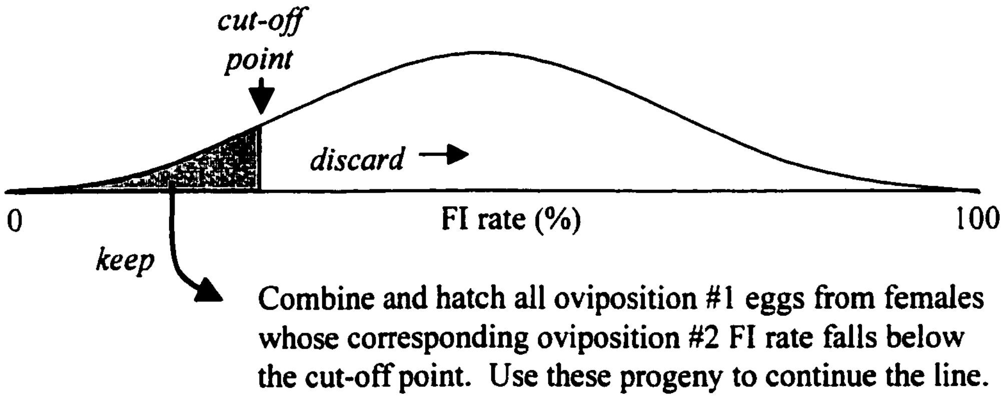

Figure 2.1. Protocol for A) bloodfeeding *Ae. triseriatus* with LAC virus and B) family-based selection on FI rate.

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(Beaty and Thompson, 1978). Distinct fluorescence foci in tissues indicated viral infection.

Family-based selection scheme. Only progeny from infected mothers were used for selection. This approach ensured that the selection focussed on potential barriers to ovarian infection rather than barriers to disseminated infection. On average, 98% of engorged mothers had a disseminated infection.

For each female, progeny from the second ovarian cycle were reared, by family, to the second or third instar and assayed for infection. The FI rate then was determined for females with 10 or more progeny. Based on the distribution of FI rates for each mosquito strain, a cut-off point for selection was chosen. This cut-off point was a function of applying a reasonably strong intensity of selection while at the same time retaining enough individuals to continue the experiment. First ovarian cycle eggs from females whose FI rate fell within the desired selection percentile were hatched, pooled, and used to continue the selection process (Figure 2.1B).

Prior to the experiment, I assayed, by FA microscopy, several hundred individuals of both sexes that were produced in the first gonotrophic cycle and arose from mothers with disseminated infections. All of these offspring were negative. Additionally, during the course of the experiment, for each colony, for each generation, after mating took place and females were removed to individual oviposition cartons, a sample (~10%) of the males was tested for LAC virus infection in the same manner. These, too, were always negative. Therefore, the founding members of each generation were uninfected.

Fisher's single-locus selection model. Based upon my selection results I inferred a model involving a single locus with a dominant allele *A* that confers TOT permissiveness

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and a recessive allele *a* that confers refractoriness (see Results). To test this inference, I attempted to fit my results to Fisher's single-locus selection model (Fisher, 1958):

$p_{t+1} = p(pw_{AA} + qw_{Aa})/\bar{w}$ and $q_{t+1} = q(pw_{Aa} + qw_{aa})/\bar{w}$ [1].

Where: p = frequency of A,

q = frequency of a and

$\bar{w}$ (the average fitness of the population) $= p^2 w_{AA} + 2pqw_{Aa} + q^2 w_{AA}$.

This model predicts the frequencies of two alleles at time $t+1$ as a function of their frequency at time t and the relative fitnesses of the three genotypes. For each round of selection, the relative fitnesses of the TOT permissive phenotypes (genotypes *AA* and *Aa*) were denoted as w_{AA} and w_{Aa}, and that of the TOT refractory phenotype (genotype *aa*) as w_{aa}. The most fit phenotype was assigned a value of 1.0. Relative fitness coefficients were determined based on the proportion of individuals of each phenotype that were selected to found the next generation. Permissive phenotypes were assumed to consist in equal parts of *AA* and *Aa* genotypes.

C. Results

To verify that my viral infection assay was sufficiently sensitive to detect the lower levels of virus typical of early instars (Beaty and Thompson, 1976), I assayed transovarially infected mosquitoes by developmental stage. No appreciable difference in infection rate was observed between instars (Table 2.1).

The results of selection through 2 generations in the Holmen strain and 3 generations in the AIDL strain are shown in Figure 2.2. There was a pronounced response to

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Table 2.1 LAC virus infection rates in various developmental stages of trans-ovarially infected *Ae. triseriatus*

	percentage infected [a]
1st instar	9
2nd instar	13
3rd instar	11
4th instar	10
pupa	9
adult male	12
adult female	13

[a] The progeny of ~200 females with disseminated infections were pooled and 100 individuals sampled per developmental stage; eggs were not assayed. Goodness-of-fit $\chi^2 = 1.64$, 6 d.f., $P = 0.95$.

selection for a low FI rate in the Holmen strain, but no response for an increased FI rate in the AIDL strain. The average FI rate in the Holmen strain, initially 18%, dropped to 5% after one generation of selection, and decreased to 3% after a second round of selection. TOT rates showed a commensurate decline in the Holmen strain, from an initial rate of 72% in the parental generation to 31% in the F_1, to 14% in the F_2. By contrast, there was no discernable response to selection for an increased FI rate in the AIDL strain. The average FI rate fluctuated from 40% in the parental generation, to 25% in the F_1, 35% in the F_2, and 31% in the F_3. Moreover, the frequency distribution of FI rates in the F_1 through F_3 generations showed no increase in rightward skewness. However there was a discernable response to selection for increased TOT rate. Despite an initial decrease in TOT from 69% to 59% (discussed below), TOT rate increased from 86% to 92% in subsequent generations.

The Holmen and AIDL strains used in this study had each been through many generations in the laboratory prior to this experiment. I recognize the possibility that

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Figure 2.2 Response to selection on FI rate in the Holmen and AIDL strains of *Ae. triseriatus*. The Holmen strain was selected for refractoriness through two generations. The AIDL strain was selected for permissiveness through two generations and then two F_3 strains were selected; one for with increased permissiveness and another for refractoriness. The first column in each histogram contains mothers with 0% FI rate; all other columns comprise 10% intervals. Between each generation, the strength of selection is indicated. For example, in the P_1 generation of the Holmen strain, progeny from mothers whose FI rates $\leq$ 20% were selected to found the F_1 generation and the average FI rate of these mothers was 6%.

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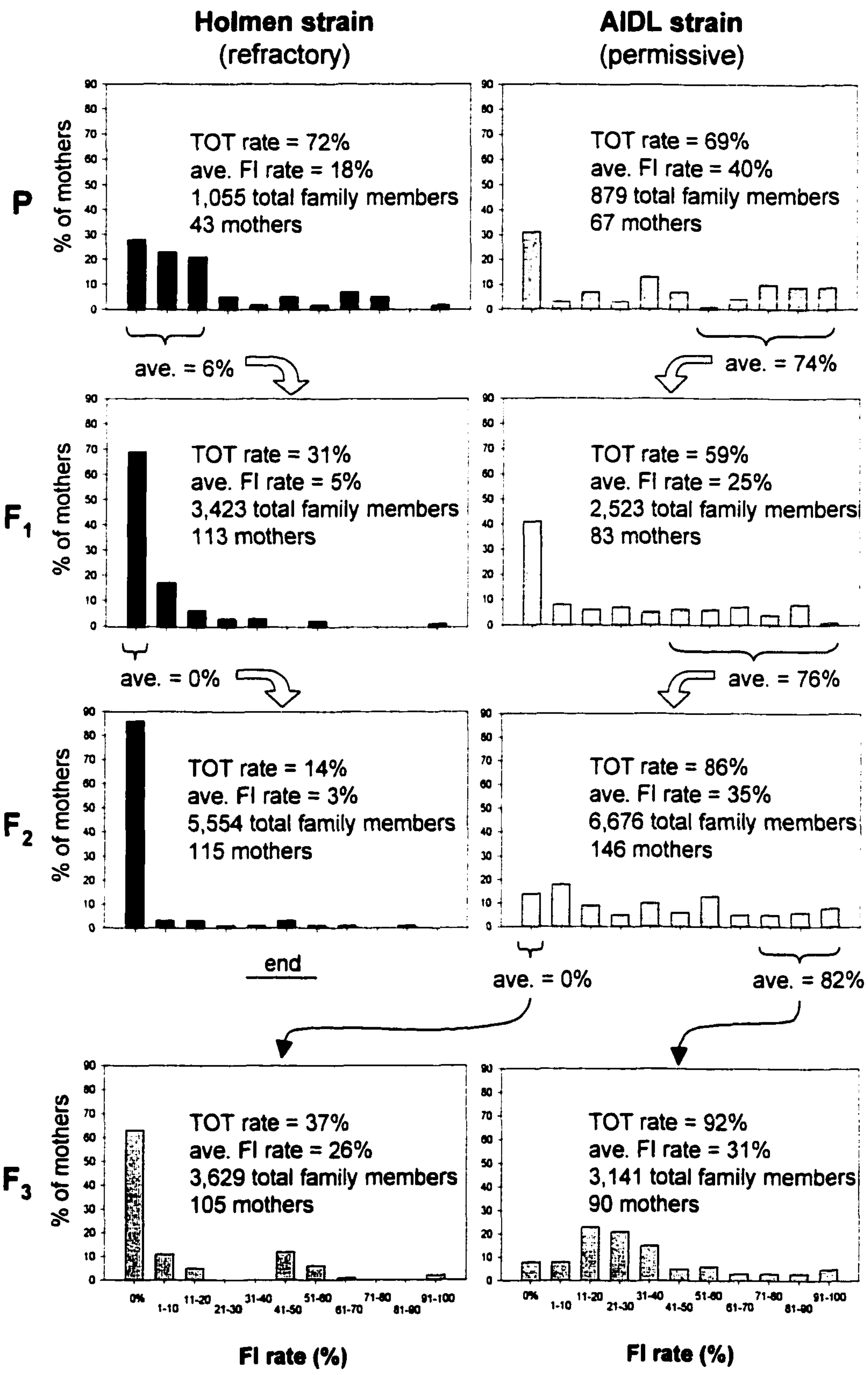

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different TOT relevant alleles at one or more loci may have reached fixation in each mosquito strain prior to the experiment, thus confounding my interpretation of the selection response. Therefore, the F_2 generation of the AIDL strain was selected for FI rate in both directions. I hypothesized that if allele fixation were not a factor, bi-directional selection in a single strain would yield similar results to those of unidirectional selection in separate strains. There was a significant response in the TOT rate, but no appreciable response in average FI rate (Table 2.2; Figure 2.2, F_3 generation).

Table 2.2 Response to selection on TOT rate.

	χ^2 value[a]	
	Holmen	AIDL
P_1 to F_1	22.58***	1.478
F_1 to F_2	8.49**	21.83***
F_2 to F_3	--	2.81

[a] Goodness-of-fit test (9 d.f. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

The strong response to selection in the Holmen strain supports a model in which TOT is conditioned by a single gene of major effect. To further test this model, I compared my observed results with those predicted by equation [1]. The equation predicts the frequencies of refractory and permissive phenotypes in each strain in response to the selection imposed on each generation. With the exception of the F_1 generation (discussed below), the observed frequencies generally fit those expected under a single locus model (Figure 2.3).

Tests of significance for response to selection on FI rate in permissive individuals are shown in Table 2.3. For the Holmen strain, in the P_1 and F_1 generations there was an excess of individuals in the lowest deciles, as would be expected when selecting for TOT

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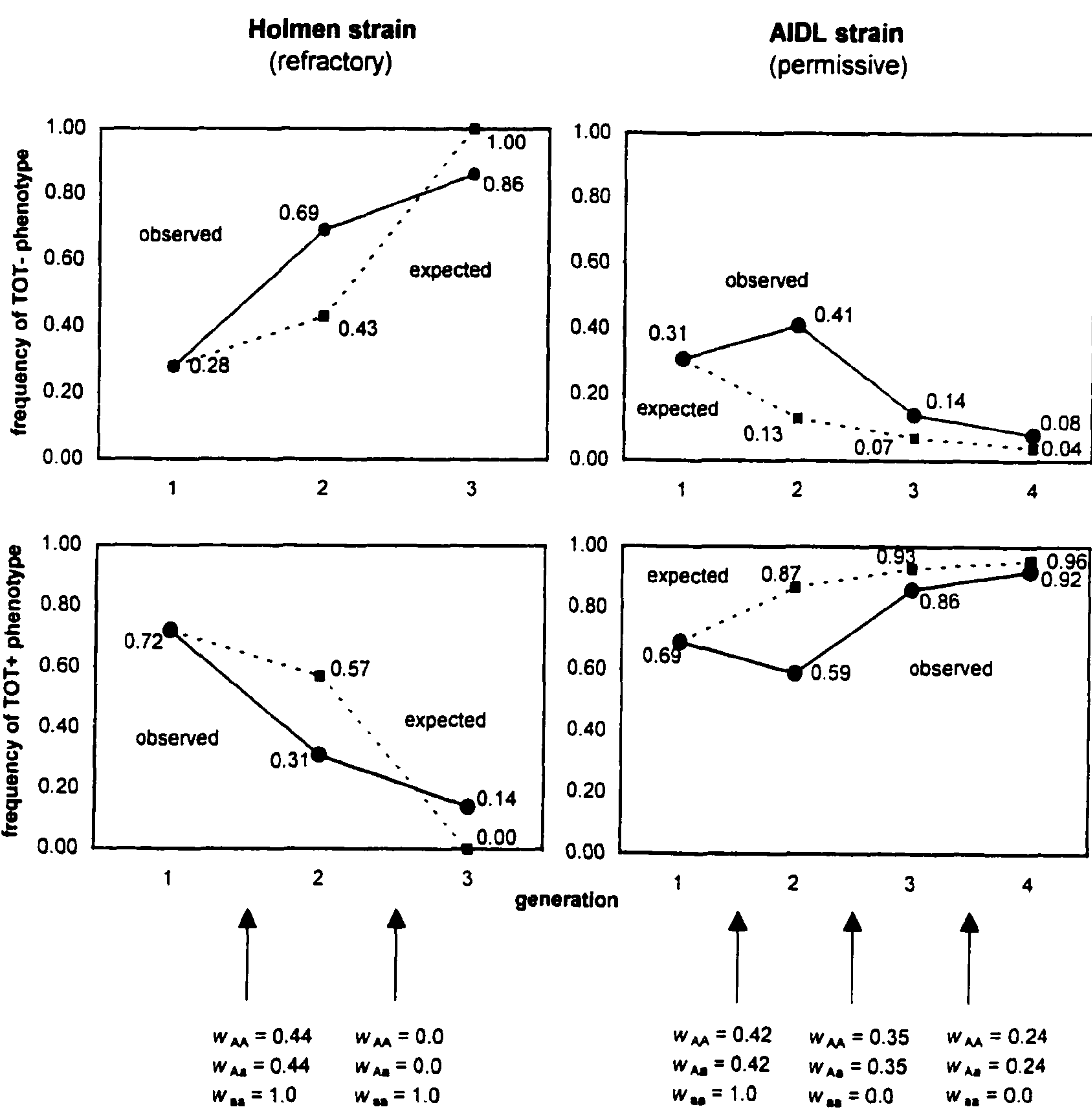

Figure 2.3 Observed (solid lines) and expected (dashed lines) changes in the frequency of TOT permissive and TOT refractory individuals in response to selection. Expected values were calculated using equation [1]. Relative fitness of each genotype, corresponding to the strength of selection (see Figure 2.2), is indicated for each generation.

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Table 2.3 Number of females in each non-zero FI rate decile in Figure 2.2 (parentheses = as a percentage of the total number of transmitting females in a given generation). The χ^2 analysis is a goodness-of-fit test to a uniform distribution. Expected values in a uniform distribution are the total number of TOT females/10. Bolded, underlined values in AIDL strain indicate deciles which caused the significant χ^2 values.

Holmen strain												
	1-10%	11 – 20	21 – 30	31 – 40	41 – 50	51 – 60	61 – 70	71 – 80	81 – 90	91 - 100	total	χ^2 value[a]
P_1 % total	10 (32.26)	9 (29.03)	2 (6.45)	1 (3.23)	2 (6.45)	1 (3.23)	3 (32.26)	2 (6.45)	0 (0.00)	1 (3.23)	31	35.13***
F_1 % total	19 (54.29)	7 (20.00)	3 (8.57)	3 (8.57)	0 (0.00)	2 (5.71)	0 (0.00)	0 (0.00)	0 (0.00)	1 (2.86)	35	88.71***
F_2 % total	4 (23.53)	4 (23.53)	1 (5.88)	1 (5.88)	4 (23.53)	1 (5.88)	1 (5.88)	0 (0.00)	1 (5.88)	0 (0.00)	17	14.18
AIDL strain												
	1-10%	11 – 20	21 – 30	31 – 40	41 – 50	51 – 60	61 – 70	71 – 80	81 – 90	91 - 100	total	χ^2 value[a]
P_1 % total	2 (4.35)	5 (10.87)	2 (4.35)	9 (19.57)	5 (10.87)	1 (2.17)	3 (6.52)	7 (15.22)	6 (13.04)	6 (13.04)	46	12.70
F_1 % total	7 (14.29)	5 (10.20)	6 (12.24)	4 (8.16)	5 (10.20)	5 (10.20)	6 (12.24)	3 (6.12)	7 (14.29)	1 (2.04)	49	6.31
F_2 % total	**27** (21.43)	13 (10.32)	8 (6.35)	14 (11.11)	9 (7.14)	19 (15.08)	8 (6.35)	7 (5.56)	9 (7.14)	12 (9.52)	126	27.81**
F_3 % total	6 (8.00)	**18** (24.00)	**17** (22.67)	12 (16.00)	4 (5.33)	5 (6.67)	3 (4.00)	3 (4.00)	3 (4.00)	4 (5.33)	75	41.93***

[a] 9 d.f. (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$)

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refractoriness. The lack of significance for the Holmen F_2 generation reflects the low number of permissive females. In the P_1 and F_1 generations of the AIDL strain FI rate was uniformly distributed. The significant χ^2 values obtained for the F_2 and F_3 generations are due mainly to the large numbers of females in several of the lower FI rate deciles (shown in bold and underlined in Table 2.3). The fact that these larger-than-average deciles occur toward the lower rather than upper range of FI rates was unexpected given that I had selected for high FI rates.

We further tested these patterns by performing a reciprocal cross between the Holmen F_2 and AIDL F_3 strains, and assaying the female progeny for TOT. Under the single gene model, where refractoriness is conditioned by recessive alleles, distributions of FI and TOT would be similar to those of the permissive strain and progeny would be 100% heterozygous at the TOT locus and uniformly TOT permissive. This is what I observed (Figure 2.4).

D. Discussion

Our selection results indicate that TOT of LAC virus in *Ae. triseriatus* has a significant heritable component. The strong response to selection in the Holmen strain indicates that a single gene of major effect conditions TOT. The pronounced response to selection in the downward direction indicates that the TOT refractory phenotype is caused by a homozygous recessive genotype, whereas permissiveness to TOT is mediated by the presence of at least one dominant allele. The strong response to selection for refractoriness occurs because the genotype of a female with a 0% FI rate is unambiguous (i.e. homozygous recessive). By contrast, because both homozygous dominant and

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Figure 2.4 FI rate distributions of female progeny resulting from a reciprocal cross between refractory and permissive selected lines. Due to decreasing fitness in the strains at this stage in the experiment, many families experienced noticeably reduced hatch rates. For comparison, I analyzed data from all mothers (distributions C and D), as well as data only from mothers producing 10 or more progeny (distributions E and F). The distributions of F_1 TOT rates were compared to those of the parents. As expected, progeny distributions were not significantly different from the distribution in the permissive (AIDL) strain, but were significantly different from the distribution in the refractory (Holmen) strain. (2 x 2 contingency χ^2: Distribution A vs. distribution B: $\chi^2 = 126.85$, $P < 0.001$; A vs. C: $\chi^2 = 79.67$, $P < 0.001$; A vs. D: $\chi^2 = 107.62$, $P < 0.001$; B vs. C: $\chi^2 = 0.004$, $P = 0.951$; B vs. D: $\chi^2 = 1.006$, $P = 0.316$; C vs. D: $\chi^2 = 0.861$, $P = 0.354$; C vs. E: $\chi^2 = 0.918$, $P = 0.565$; D vs. F: $\chi^2 = 0.074$, $P = 0.916$).

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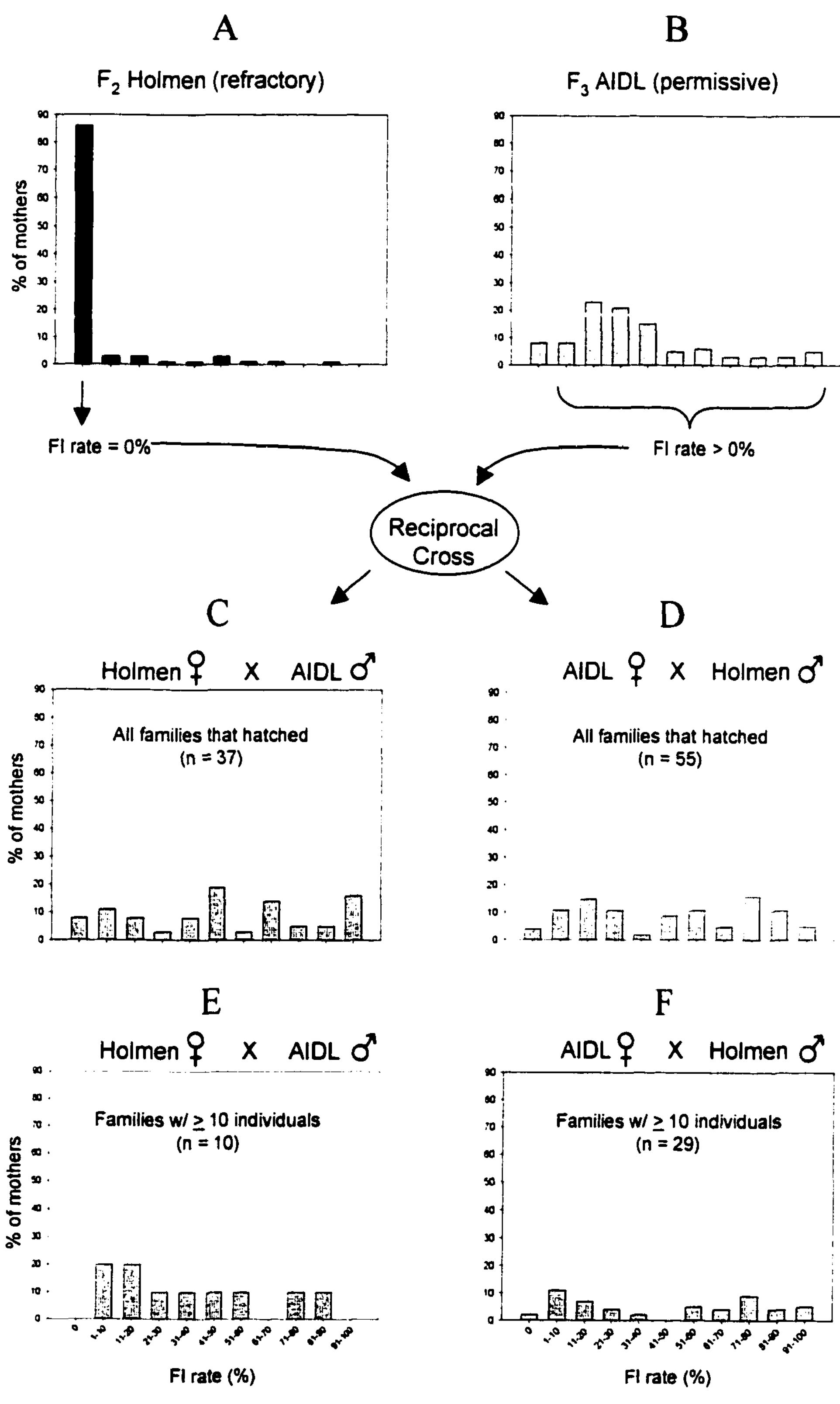

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heterozygous genotypes had indistinguishable (permissive) phenotypes, when selecting for permissiveness I inevitably chose some individuals that were heterozygous. When these individuals mated, there was a 25% probability of obtaining homozygous recessive (refractory) progeny, hence the continual appearance of TOT refractory mothers. In light of this, the anomalous increase of refractory F_1 mothers in the permissive strain (Figure 2.2) probably resulted from having used founding P_1 stock comprised of an inadvertently large proportion of heterozygotes. Excesses of heterozygotes maintained by the presence of closely linked deleterious and lethal alleles have been documented in colonized *Aedes* species in general (Munstermann, 1994) and particularly in *Ae. triseriatus* (Matthews and Craig, 1987; 1989).

Heterozygote excess may also explain the discrepancies between observed and expected phenotype frequencies in this strain using equation [1] (Figure 2.3). The subsequent two generations of the permissive strain show the expected decrease in refractory females. With repeated upward selection on the permissive strain, I would expect refractory females to eventually decrease to near zero.

The inheritance of alleles controlling viral infection of oocytes in *Ae. triseriatus* fits a model in which TOT dominant alleles encode a membrane protein that acts as a viral receptor on or within the ovaries. Proteins encoded by the dominant allele effectively bind LAC virus allowing infection to reach the follicles while homozygous recessive individuals would lack the viral receptor. However, while specific receptors have been described for some arboviruses, for example the laminin receptor for Sindbis virus (Wang *et al.*, 1991) and phosphatidylserine for vesicular stomatitis viruses (Schlegel *et al.*, 1982), receptors for LAC virus have yet to be described. Given the appearance of some

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transmitting females in the F_2 generation of the Holmen strain, a generation that was founded entirely by progeny from refractory females, the absence of a functional receptor may nonetheless permit a small amount of "leakage" of virus into the ovaries possibly due to disruption of the integrity of the ovarian sheath during the first gonotrophic cycle.

Alternatively, it is possible that infection of progeny in this case could have occurred by transovum transmission whereby infection occurs late in development as eggs pass through the oviducts and calyx during oviposition. If these structures contain large amounts of virus, infection of the egg can occur since the chorion and micropyle apparatus are still permeable at this stage (Clements, 1992). The vertical transmission of yellow fever virus in *Ae. aegypti* was shown to occur by this mechanism (Rosen *et al.*, 1989). Given that large amounts of LAC virus have been detected in the calyx of *Ae. triseriatus* with disseminated infections (Chandler *et al.*, 1998), it is possible that females refractory to TOT may nonetheless infect some of their progeny by transovum transmission. Finally, the appearance of infected progeny could also be due to the influence of other genes of lesser effect.

The absence of a response to selection on FI rate indicates that this parameter is not under genetic control. It would appear as though, for each female, the number of follicles that become infected during TOT is dictated by non-Mendelian, stochastic factors that could have potentiating or inhibiting effects on viral infectivity or replication. These may include factors in the mosquito such as variation in the volume of cytoplasm, the relative locations of ovarioles in the ovary, relative concentrations of soluble factors in individual oocytes, or point of infection entry into an ovary. These may also include random factors affecting replication, assembly, and survival of LAC virus in ovarian tissues. This might

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explain why, despite strong selection in either direction, FI rates continued to range from 0% to 100%. This stochastic component also would have the effect of masking any additive gene effects that may exist: one functional copy of the gene could give rise to as many infected follicles as two functional copies.

In summary, with regard to the vertical transmission of LAC virus in *Ae. triseriatus*, the salient parameter responding to selection is the TOT and not the FI rate. The chief distinction to be made regarding the FI rate distributions is between TOT refractory versus permissive mothers. These results argue for caution in assigning significance to correlations observed between FI rate and other parameters of interest without taking into consideration the stochastic nature of FI rate. Finally, in light of these results, future experiments of this kind might be modified in such a way as to assay families for infection by pools, thereby focusing strictly on TOT. A protocol of this kind would allow one to assay wild populations of *Ae. triseriatus* for the frequency of the recessive allele, thereby obtaining a rapid assessment of TOT potential in these populations.

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Chapter 3

A RAPD-SSCP Linkage Map for the Eastern Treehole Mosquito, *Aedes triseriatus*, and Identification of Quantitative Trait Loci Affecting Sex Ratio.

A. Introduction

The eastern treehole mosquito [*Aedes* (*Protomacleaya*) *triseriatus* (Say)] is the principal vector of La Crosse (LAC), virus the leading cause of pediatric arthropod-borne viral (arboviral) encephalitis in the United States (Calisher, 1994). Variation in traits relating to the vector competence of (i.e. the ability to transmit) LAC virus has been described for different strains of this species. Studies have documented variation in 1) survival of LAC-infected embryos during diapause (McGaw *et al.*, 1998), 2) adult susceptibility and ability to orally transmit LAC virus (Grimstad *et al.*, 1977), 3) permeability of anatomical barriers to dissemination (Paulson *et al.*, 1989), and 4) ability to transovarially transmit LAC virus (Graham *et al.*, 1999a). However, the inheritance and genetic control of these traits are not well understood, due in part to the lack of a high resolution linkage map for the analysis of complex traits in this species. Indeed, among insects in general there remains a paucity of high resolution linkage maps, due largely to the tedious mapping procedures used in the past (Heckel, 1993).

In recent years, the development of molecular techniques that reveal large amounts of genetic variation at individual loci has made it possible to map hundreds of genetic markers simultaneously in a single cross. When combined with the analysis of

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complex traits, intensive multi-point linkage mapping allows the identification of the number and effects of genes underlying phenotypic characters (quantitative trait loci, QTLs). This new class of markers includes restriction fragment length polymorphisms (RFLPs, Botstein *et al.*,1980), simple sequence repeat loci (SSR microsatellites), and random amplified polymorphic DNA (RAPD, Williams *et al.*, 1990).

In the yellow fever mosquito (*Aedes aegypti*) RFLPs have been used to map QTLs affecting vector competence for filarial worms (Severson *et al.* 1994) and malaria parasites (Severson *et al.* 1995a). Microsatellites have been used to map QTLs affecting *Plasmodium* encapsulation in *Anopheles gambiae* (Zheng *et al.* 1997). RAPD PCR offers several advantages over the use of RFLPs and microsatellites in that it does not require genomic library construction, sequencing, or expensive design and construction of primers. Despite problems with the dominance of RAPD polymorphisms, genetic linkage maps have been constructed with RAPD markers for a wide variety of species including trees (Byrne *et al.*, 1995, Krutovskii *et al.*, 1998), several agricultural crop species (Echt *et al.*, 1993; Kesseli *et al.*, 1994; Weide *et al.*, 1993), the zebrafish (Postlethwait *et al.*, 1994), two monkeyflower species (Bradshaw *et al.*, 1995), the honeybee (Hunt and Page, 1995a), the silkworm (Promboon *et al.*, 1995; Yasukochi, 1998), *Ae. aegypti* (Antolin *et al.*, 1996) and the Asian tiger mosquito (*Ae. albopictus*, Mutebi *et al.*, 1997). Similarly, RAPD markers have been used to map QTLs affecting complex traits in a variety of species. Among insects these include sex determination, foraging and stinging behaviors in the honey bee (Hunt and Page, 1994,1995b; Hunt *et al.*, 1998), and vector competence of *Ae. aegypti* for Dengue-2 virus (Bosio *et al.*, 1999b).

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In this study I construct a multi-point linkage map for *Ae. triseriatus* derived from RAPD fragments subjected to single strand conformation polymorphism (SSCP) analysis. Construction of a RAPD-SSCP linkage map for *Ae. triseriatus* allowed me to examine the recombinational map length in relation to genome size (Black and Rai, 1988). These two parameters, combined with cytological observations (Rao and Rai, 1987a), provide a glimpse into genome structure and evolution in this species.

Over the course of this and other experiments, I consistently observed significant female-biased sex ratios in the *Ae. triseriatus* strains in our insectary. A female-biased sex ratio was observed in two separate strains in a total of 64 families (Figure 3.1). In culicine mosquitoes, sex is determined by genes at a single locus; females are homozygous recessive (*mm*) while males are heterozygous for a dominant allele (*Mm*)(Gilchrist and Haldane, 1947). Thus, the male parent determines sex ratio in the progeny and normal segregation results in roughly equal numbers of males and females. Genetic mechanisms causing a departure from this theoretical expectation have been documented in several culicine species. In some strains of *Ae. aegypti*, male-biased sex ratios are caused by interaction of a Distorter (*D*) gene, linked to *M*, and variants of *m* that are susceptible to *D* (Hickey and Craig, 1966). An additional locus, *t*, has been shown to confer tolerance to *D* (Wood and Ouda, 1987). In *Culex pipiens* male-biased sex ratios have been ascribed to a similar interplay of dominant (*MD*) and susceptible recessive (m^s) alleles (Clements, 1992; Sweeney and Barr, 1978).

Female-biased sex ratios in culicines have been described in the context of thermal stress during embryogenesis (Horsfall and Anderson, 1963, 1964, 1965), and differential larval mortality (Danks and Corbet, 1973). Also, in several *Aedes* species,

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feminization of genetic males was observed to result from high temperatures (Brust, 1968; Horsfall, 1974) and developmental abnormalities associated with interspecific crosses (Hilburn and Rai, 1982). However, genetic analyses of mechanisms underlying female-biased primary sex ratios in the Culicidae are lacking. In this study I test the hypothesis that the female-biased sex ratios I observed are conditioned by loci in addition to the sex locus. I treat sex as a quantitative trait in *Ae. triseriatus* to ascertain the presence and location of any QTLs with a significant effect on this phenotype. The presence of a single QTL would suggest that environmental factors in the laboratory cause the consistent differential survival or development of females, whereas identification of multiple independent loci would indicate that a female-biased sex ratio arises from genes that differentially affect survival and possibly development of the sexes.

B. Materials and Methods

***Aedes triseriatus* strains.** The AIDL and Holmen strains of *Ae. triseriatus* originated from eggs collected at different sites near La Crosse, Wisconsin. The AIDL strain originated in 1983 and has been in colony for ~ 30 generations, whereas the Holmen strain, established in 1992, has been through 15 generations. Each colony was maintained at 20-23°C, 75% relative humidity, and a photocycle of 16:8hr L:D with a 60 min crepuscular period at dawn and dusk. Larvae were fed Tetra-Min® fish food. Adults were housed in 24 x 24 x 24 in flight cages and consisted of 500 – 1,000 individuals per strain.

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Females from the AIDL and Holmen strains were allowed to mate, blood feed on mice, then were removed to individual 1-pint oviposition containers. Eggs were oviposited on strips of moist paper towel. Sex ratios in 64 families (Figure 3.1) were calculated among individuals reaching adulthood (average number of individuals/family = 36).

Mating scheme. *Ae. triseriatus* is a swarm-breeding mosquito (Wright *et al.*, 1966). Copulation and insemination occur only in the presence of numerous males, thereby precluding single pair mating. My attempts at artificially induced copulation were unsuccessful, therefore paternal RAPD profiles were inferred from the maternal genotype and marker segregation ratios in the offspring. Female mosquitoes mate only once (Craig, 1967), thus precluding the possibility of multiple paternal genotypes among progeny. Experiments aimed at maximizing insemination rates in *Ae. triseriatus* females with a minimum number of males revealed an optimum of 10 to 20 males per 5 females and a minimum cage size of 1 cubic foot (data not shown).

Linkage mapping was performed on the AIDL strain using RAPD-SSCP markers segregating in an F_2 intercross. Five virgin females were placed in a 1-gallon carton with twenty males, allowed to mate over five days, then fed on a mouse. Males were collected and frozen for genotyping. Females were removed from the carton and individually placed in 1-pint oviposition containers with mesh fabric lids. Eggs were oviposited on strips of moist paper towel. Following oviposition, each female was collected and frozen. Eggs from each female were hatched separately to produce five separate F_1 families. Each F_1 family was allowed to interbreed and females were fed upon a mouse. Males were collected and frozen for genotyping. Each F_1 female was removed to an oviposition

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carton. In order to obtain a large F_2 family for mapping, multiple gonotrophic cycles were induced by bloodfeeding in each F_1 female prior to removal and freezing. This was done by allowing them to feed on hanging droplets consisting in equal parts of 10% sucrose solution and defibrinated sheep blood (Colorado Serum Co., Denver, CO). Several candidate F_2 families were hatched, reared and frozen. The family chosen for mapping consisted of 146 individuals and had a significantly female-biased (106/146 = 73%; $\chi^2 = 29.8$, 1 d.f., $P < 0.001$) sex ratio.

DNA isolation and RAPD-PCR. DNA was extracted following the protocol of Bender *et al.* (1983). DNA was resuspended in 1 ml TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0). The majority of the DNA was archived at –80°C; DNA to be used immediately for PCR reactions was stored at 4°C. RAPD-PCR was performed using each of eight primers (Operon Technologies, Alameda, California). These were A06 (5' GGTCCCTGAC 3"), A09 (5' GGGTAACGCC 3'), A20 (5' GTTGCGATCC 3'), B19 (5' ACCCCCGAAG 3'), B20 (5' GGACCCTTAC 3'), C04 (5' CCGCATCTAC 3'), C13 (5' AAGCCTCGTC 3'), and C16 (5' CACACTCCAAG 3'). PCR was performed in 96 well plates on a thermal cycler (PTC-100, MJ Research). A single large PCR reaction mixture sufficient to perform 100 50 μl reactions was made by mixing 4,350 μl dd H_2O, 500 μl 10X Taq buffer (500 mM KCl, 100 mM Tris-HCL pH 9.0), 50 μl of 20 mM dNTPs, and 500 pm of the primer. This reaction mixture was set under a UV light source (302 nm) for 10 min, after which 10 μl of Taq polymerase were added. The mixture was then dispensed into a 96 well plate. Two μl of template DNA were added to each well, followed by a drop of sterilized mineral oil. Each set of PCR reactions (i.e. one 96 well plate) was checked for contamination by the use of a negative control containing all

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reagents except template DNA. The plate was then subjected to 45 cycles of (1) 95°C for 1 min (denaturation); (2) 35°C for 1 min (annealing); (3) ramp to 72°C at a rate of 1°C every 8 s; and (4) 72°C for 2 min (extension). A final 72°C extension was carried out for 7 min and the temperature was held at 4°C. Samples were stored at 4°C until electrophoresis. The contents of each well were tested for the presence of amplified products by loading 5 µl from each well onto a 1.5% TBE agarose gel, performing electrophoresis for 15-20 min at 112 V, staining with ethidium bromide, and viewing the gel over a UV transilluminator.

Electrophoresis to detect SSCPs. SSCP analysis and silver staining were performed according to the protocols described by Hiss *et al.* (1994), Antolin *et al.* (1996), and Black and Duteau (1997). Briefly, PCR products (2.5 µl out of 50 µl) were mixed with 4.5 µl of denaturing loading buffer and electrophoresed on large (40 X 50 cm), thin (0.4 mm) glycerol (5%) polyacrylamide (5%, 2% cross-linking) gels. Electrophoresis proceeded at constant current (16 mA) at room temperature for 16 hr (overnight). DNA fragments were detected with silver staining following Black and DuTeau (1997).

Scoring of RAPD-SSCP bands. Amplified markers were scored directly from dried gels by measuring band mobility relative to known size markers (1-kb ladder, BRL Laboratories). To estimate sizes of amplified DNA fragments, the reciprocal of fragment size was regressed upon the reciprocal of mobility (Schaffer and Sederoff, 1981). RAPD markers were named by the primer designation followed by a period and the estimated size of the fragment.

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The RAPD-SSCP method generates numerous bands, not all of which are repeatable. Spurious bands that appeared in only a few individuals were disregarded. Mendelian segregation was tested in the remainder of RAPD loci using the single locus analysis (JMSLA) procedure in JoinMap 2.0 (Stam and van Ooijen, 1995). Loci that failed to meet Mendelian expectations were discarded.

Linkage analysis. F_2 offspring genotypes were analyzed using JoinMap 2.0. Recombination fractions were converted to map units (centiMorgans, cM) by the Kosambi mapping function (Kosambi, 1944) using the pairwise recombination (JMREC) procedure of JoinMap 2.0. Initially, a minimal logarithm of odds (LOD) score of 3.0 was used to group markers. DRAWMAP 1.1 (van Ooijen, 1994) was used to plot a linkage map.

Mapping sex as a quantitative trait. The computer program QTL cartographer 1.12 (Basten *et al.*, 1997) was used to test for QTLs that affect sex ratio. Sex was treated as a binary trait and scored as 0 (female) or 1 (male). Initially, stepwise linear regression was used to reveal those markers most closely associated with QTLs and to quantify and rank the magnitude of each marker's phenotypic effect. Standard (Lander and Botstein 1989) and composite (Zeng, 1994) interval mapping were then performed. Standard interval mapping (SIM) estimates QTLs at only one location at a time and can give biased results due to the effects that additional QTLs contribute to sampling variance and due to the fact that when two QTLs are linked, their combined effects can yield upwardly biased estimates. Composite interval mapping (CIM) uses a combination of multiple regression and maximum likelihood methods to reduce this bias (Zeng, 1994). CIM fits regression coefficients for background markers to account for variance caused by non-

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target QTLs. The variance from other QTLs is accounted for by including partial regression coefficients from markers in other regions of the genome and thus the effects of other QTLs are not present as residual variance. Following Jansen and Stam (1994), background markers were chosen by stepwise regression using the SRmapqtl procedure in QTL Cartographer. The number of markers used to control for genetic background in CIM was 2.0 and the window size (interval) was 10 cM. Dominance effects were tested by calculating LOD scores for the comparison of the null hypothesis that there are no genetic effects with the alternative that there are dominance effects, but no additive genetic effects (H_2/H_0, Basten *et al.*, 1997).

To confirm the results of CIM, each RAPD-SSCP marker was also analyzed by a contingency χ^2 test to determine if sex is equally distributed among genotypes. The χ^2 analysis was also used to determine if QTLs affecting sex ratio arose from one or both parents (Table 3.1). Based on the consistent sex ratio bias observed among families (Figure 3.1) I anticipated that QTLs would originate from both parents. Finally, a permutation procedure was used to test the consistency with which the data set supports a particular QTL (Churchill and Doerge, 1994). Following the initial CIM analysis, the Zmapqtl procedure in QTL Cartographer was used to randomly permute the phenotypic traits and genotypes 100 times and then perform CIM in each of the 100 permuted data sets. The program then reported the proportion of permuted replicates that had LOD values larger than the original LOD.

The marker data were also analyzed using a model designed for complex binary traits (Xu *et al.*, 1999). Such traits are derived from the threshold characters of quantitative genetics. Threshold characters have an underlying continuity, termed the

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liability, with a threshold which imposes a discontinuity on the visible expression (Falconer and MacKay, 1996). Thus, there exists a fixed threshold in the scale of liability, below which an individual expresses one phenotype (e.g. refractoriness to TOT), and above which it expresses another (e.g. permissiveness). The model developed by Xu *et al.* (1999) transforms the liability to a normal distribution using a probit scale, and liability is described using a single linear model with a heterogeneous residual variance. One thousand random iterations of phenotype were performed on a fixed genotype file. In each iteration the program recorded the highest LOD score for each point and the highest LOD score in that iteration. The program generated the 950th highest value for each point (the 95% comparisonwise error rate) and the 950th highest value for each iteration (95% experimentwise error rate). This model also generated estimates of additive genetic variance and dominance variance for each putative QTL.

C. Results

Data showing the distribution of sex ratios in the AIDL and Holmen strains of *Ae. triseriatus* are shown in Figure 3.1. Collectively, a significant female-biased sex ratio was observed in a total of 64 families (Figure 3.1).

A total of 74 polymorphic RAPD-SSCP markers were identified that segregated in a Mendelian fashion, with an average of 9 markers per primer. At a minimal LOD of 2.7, the JMGRP procedure in JoinMap split the markers into two linkage groups and markers A20.391 and A09.648 could not be joined to the map. Linkage group I contained 48 markers and linkage group II contained 24 markers. At an LOD of 4.0 JoinMap split the markers into three groups: linkage group I remained intact with 48 markers while linkage

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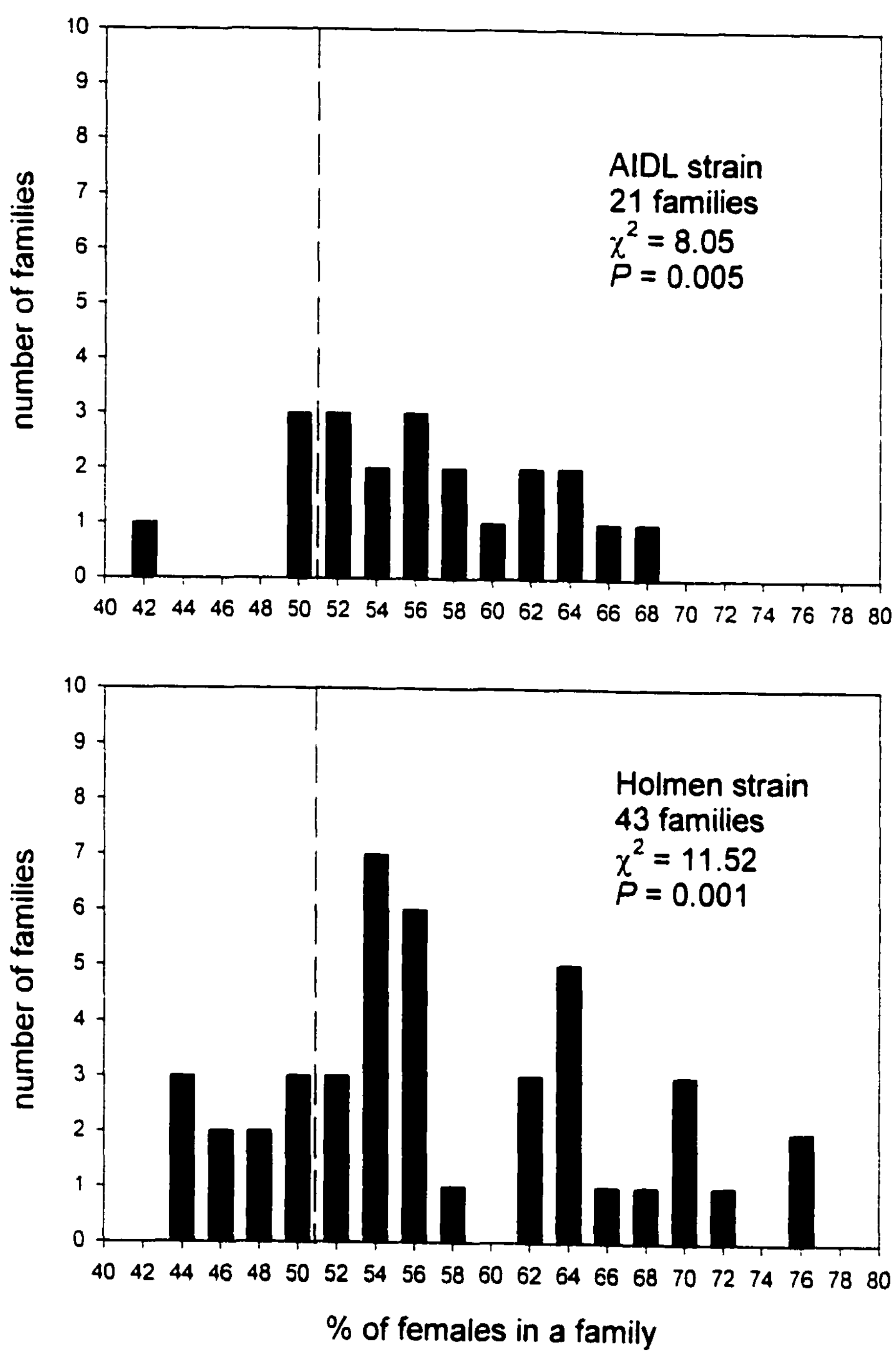

Figure 3.1. Sex ratios observed in the AIDL and Holmen strains of *Ae. triseriatus*. Chi square goodness-of-fit test is based on the null hypothesis that there are equal numbers of families above and below the threshold of 50% females (dashed line).

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group II now contained 10 markers and linkage group III contained 14 markers. At an LOD of 4.2 three markers split from linkage group I to yield a total of four groups.

Despite the increased resolving power of SSCP analysis, no codominant alleles were revealed. This is similar to the results of Mutebi *et al.* (1997) in which all 68 RAPD-SSCP loci mapped in *Ae. albopictus* segregated as band presence/absence polymorphisms. By contrast, RAPD-SSCP analysis revealed codominant alleles in *Ae. aegypti* (10 codominant alleles/94 RAPD loci,11%) and the parasitic wasp *Bracon hebetor* (36 codominant alleles/74 RAPD loci, 45%)(Antolin *et al.*, 1996).

The linkage map generated from these marker data is shown in Figure 3.2. The estimated size of individual chromosomes is 93 cM for chromosome I, 63 cM for chromosome II, and 37 for chromosome III. My designation of chromosome I is based on the observation that all QTLs affecting sex ratio map to this linkage group. The shortest linkage group was designated chromosome III, since this was the shortest *Ae. triseriatus* chromosome mapped by Munstermann (1989). The estimated length of the entire linkage map is 193 cM, with an overall resolution of one marker every 2.7 cM. The haploid genome size for *Ae. triseriatus* is 1.5×10^9 bp (1.52 pg DNA)(Black and Rai, 1988; Rao and Rai, 1987b). The relationship between genome size and recombinational map length is therefore roughly 7.9 Mbp DNA/cM.

As has been shown in other linkage studies using RAPDs (Antolin *et al.*, 1996; Hunt and Page, 1994; Mutebi *et al.*, 1997) markers amplified by the same RAPD primer had a tendency to cluster together. This could result from clustering of identical or similar repetitive regions in the genome (Williams *et al.*, 1990), or from different conformations of DNA amplified from the same locus. An example can be seen in the

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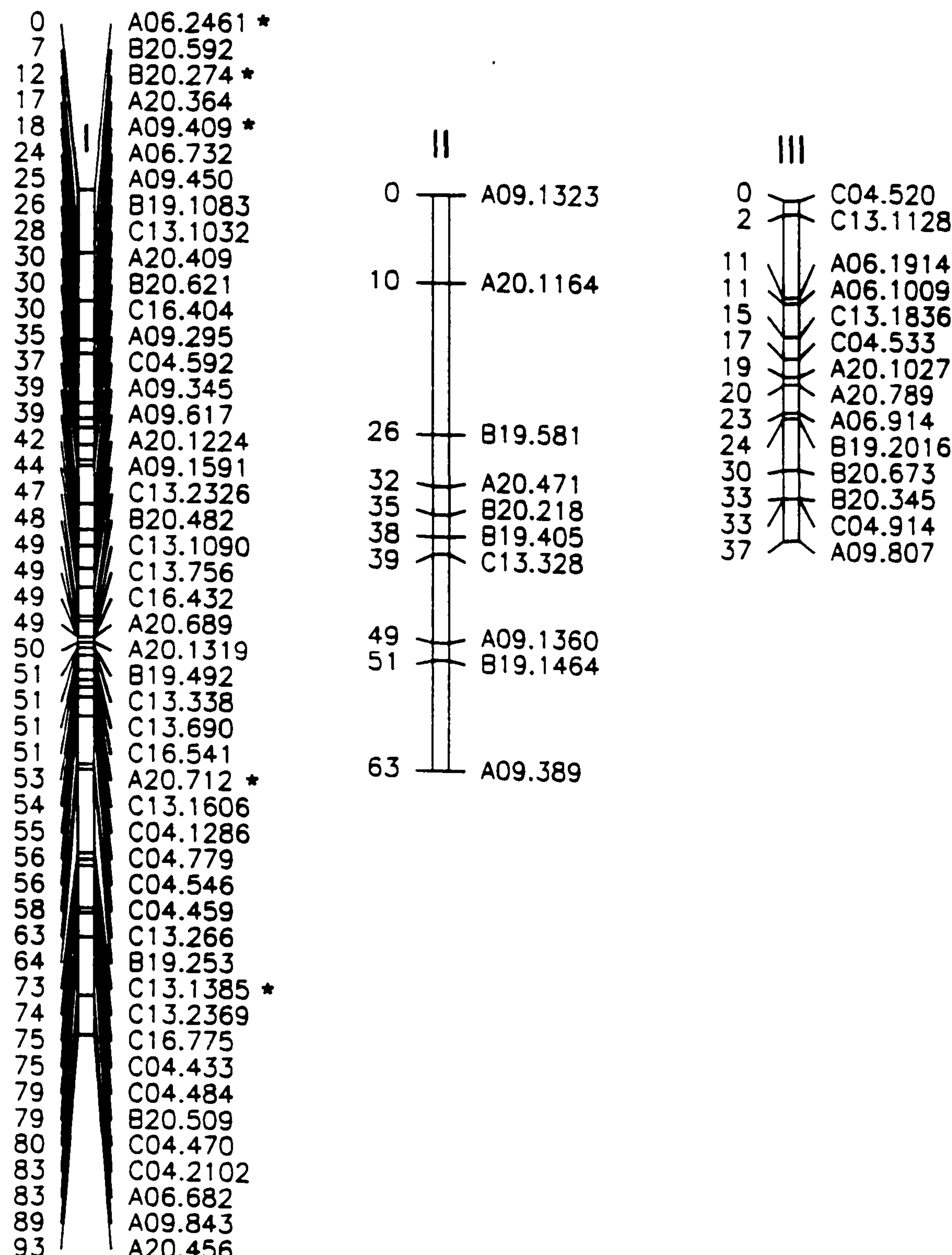

Figure 3.2. RAPD-SSCP linkage map for *Ae. triseriatus*. Map units on the left of each linkage group are in centiMorgans. Each marker is designated by the name of the RAPD primer, followed by a period, then the size in bp of the amplified fragment. Asterisks indicate markers with a significant association with sex, according to the permutation tests of Churchill and Doerge (1994).

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four closely linked markers from primer C04 on chromosome I (Figure 3.2). Different conformations of the same DNA may appear as pairs of markers with no recombination between them (e.g. A09.345 & A09.617, C04.779 & C04.546, A06.1914 & A06.1009). However, identical map distances can also result for markers corresponding to different, proximate genomic regions if there is little or no recombination between them.

Treating sex as a phenotypic character rather than a genetic marker in an *Ae. triseriatus* family with a significantly female-biased (73%) sex ratio revealed four putative QTLs on chromosome I with a significant phenotypic effect. Table 3.1 lists the χ^2 values, P_1 parentage of the male-reducing genotype, and permutation test *P* values for each marker showing a significant association with sex. Among these markers, the female-biased sex ratio phenotype is descended in roughly equal numbers from each P_1 parent. It is reasonable to assume that the QTL in the vicinity of marker B20.274 is the major sex-determining locus given its high χ^2 value and CIM LOD score (Table 3.1, Figure 3.3). Furthermore, the association of B20.274 alleles with the sex of F_2 offspring is in a direction predicted by Gilchrist and Haldane's (1947) model of sex determination in which maleness is determined by a dominant allele at the sex locus and at which females are homozygous recessive. The dominant B20.274 allele inherited from the P_1 father is associated with a large excess of F_2 males, while the recessive allele inherited from the P_1 mother is associated with an excess of F_2 females. SIM revealed the same and several additional markers with significant effect (Figure 3.4), indicating that controlling for covariance of markers was important in this analysis. The very high LOD score associated with marker A06.2461 on chromosome I is noteworthy. High LOD scores observed at the ends of chromosomes are often partially artifactual due to reduced

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Table 3.1. For each marker with a significant association with sex ratio distortion: the observed and expected numbers of F_2 individuals for each marker genotype as a function of P_1 parentage, χ^2 value and *P* value from the permutation test. (+ = RAPD-SSCP band present, - = RAPD-SSCP band absent, xs = excess, def = deficiency)

marker		RAPD-SSCP marker from P_1 mother		RAPD-SSCP marker from P_1 father		χ^2 Value[a]	*P* value from permutation test
		F_2 females (-)	F_2 males (+)	F_2 females (-)	F_2 males (+)		
A06.2461	obs.	18 (def)	16 (xs)	86 (xs)	24 (def)	8.25***	< 0.0001
	exp.	24.6	9.4	79.4	30.6		
B20.274	obs.	90 (xs)	6 (def)	15 (def)	30 (xs)	58.92***	< 0.0001
	exp.	71.5	24.5	33.5	11.5		
A09.409	obs.	67 (xs)	15 (def)	34 (def)	25 (xs)	9.79***	< 0.0001
	exp.	58.7	23.3	42.3	16.7		
A20.712	obs.	53 (xs)	11 (def)	52 (def)	26 (xs)	4.76*	0.05
	exp.	47.3	16.7	57.7	20.3		
C13.1385	obs.	67 (def)	37 (xs)	38 (xs)	3 (def)	11.76***	0.01
	exp.	75.3	28.7	29.7	11.3		

[a] (* $P = 0.05$, ** $P = 0.01$, *** $P = 0.001$),

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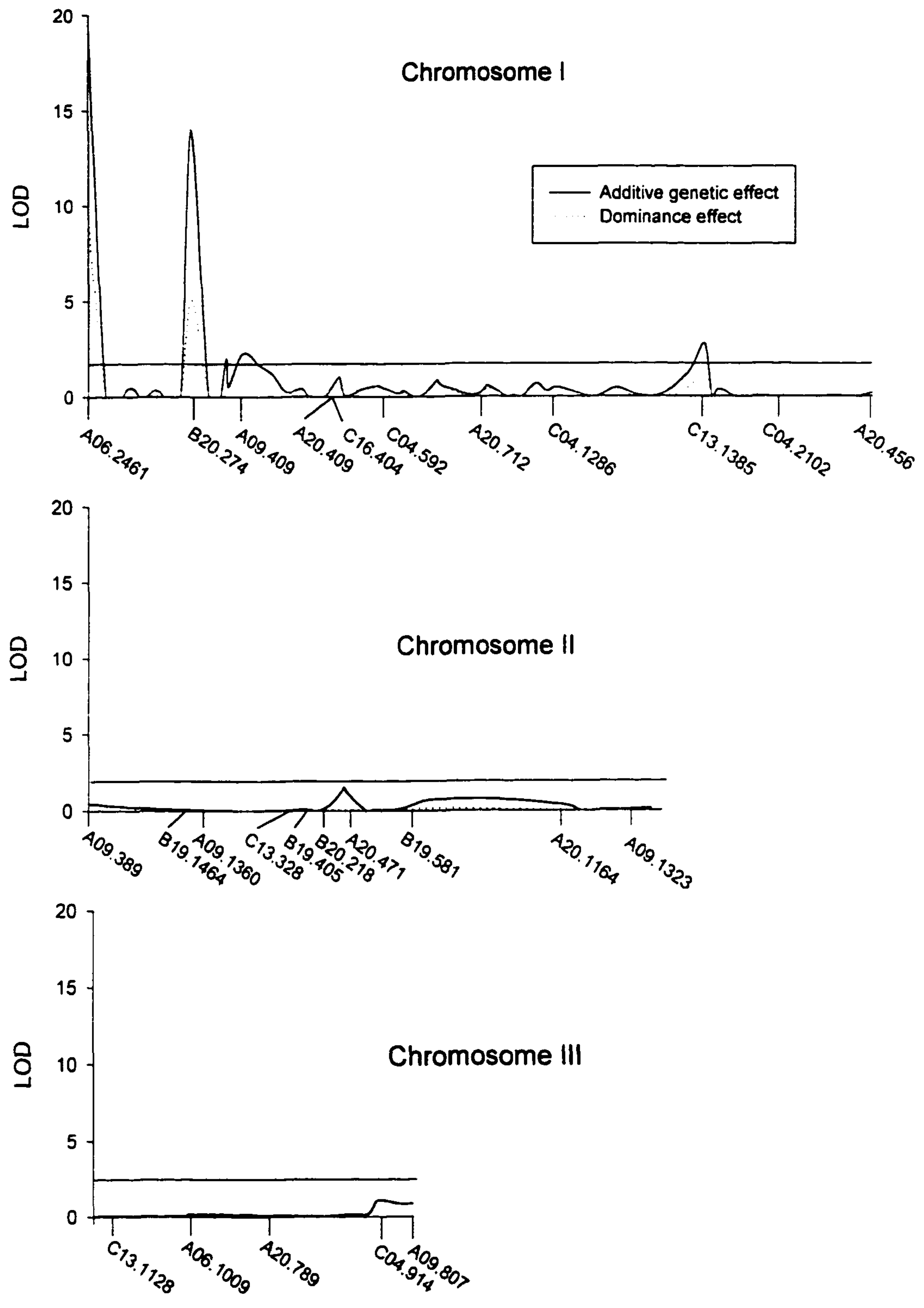

Figure 3.3 Plots of LOD scores for sex on each *Ae. triseriatus* chromosome. LOD values were estimated using composite interval mapping. The X axis of each plot is proportional to the recombinational distance shown in Figure 3.2. Locations of selected markers are shown relative to their positions on the linkage map (Figure 3.2). The threshold value shown corresponds to an LOD value of 2.

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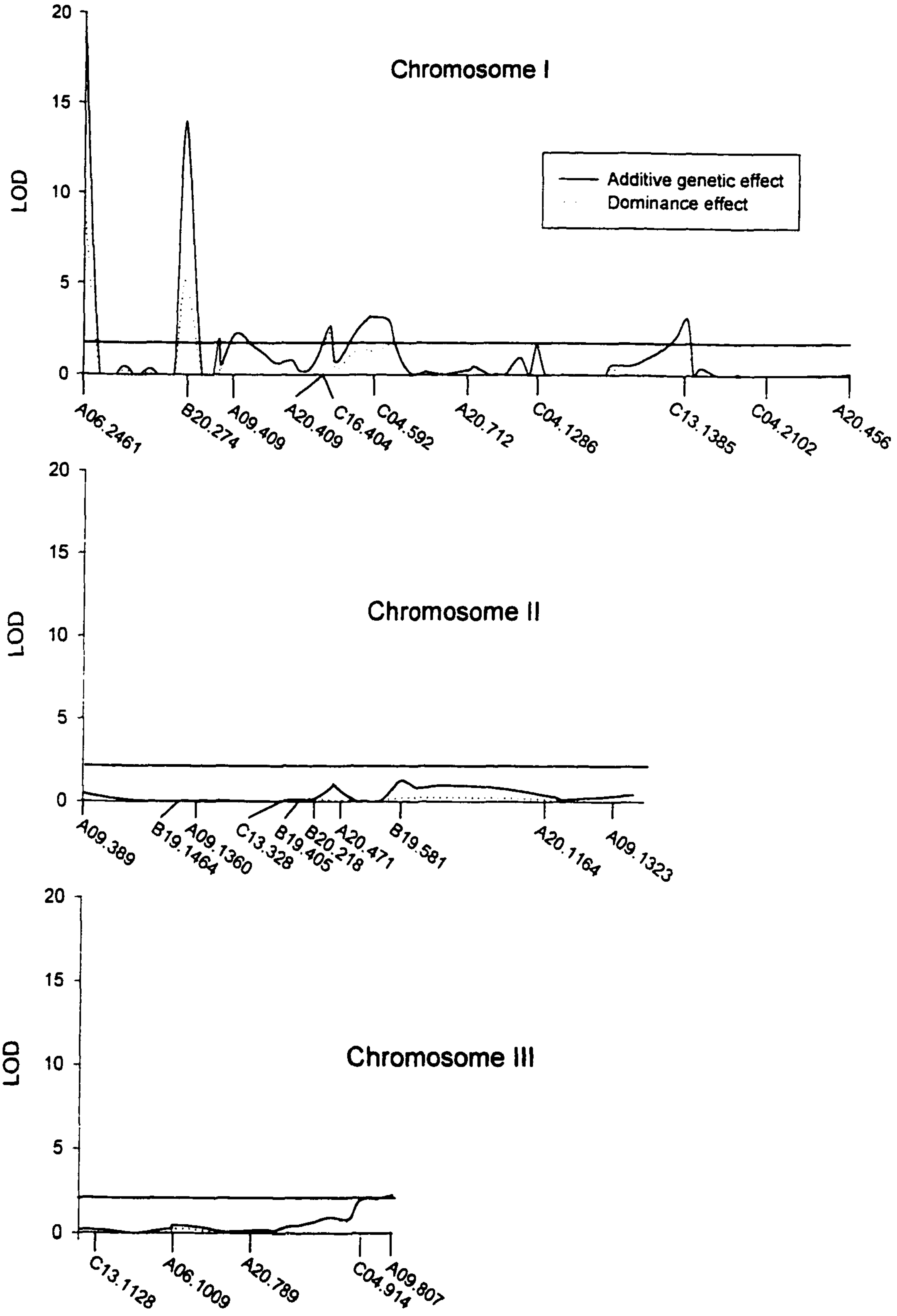

Figure 3.4. Plots of LOD scores for sex on each *Ae. triseriatus* chromosome. LOD values were estimated using standard interval mapping. The X axis of each plot is proportional to the recombinational distance shown in Figure 3.2. Locations of selected markers are shown relative to their positions on the linkage map (Figure 3.2). The threshold value shown corresponds to an LOD value of 2.

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recombination at telomeres. In such cases, adjacent markers are often included in the LOD peak. However, this is not the case with marker A06.2461. Marker B20.274, only 12 cM away from A06.2461, has its own distinct LOD peak indicating that there are probably separate QTLs at these two locations. That these two QTLs are independent is further supported by the observation that their associated markers originated from different P_1 parents. The excess-female phenotype in A20.2461 originates from the P_1 father whereas the excess-female phenotype in B20.274 originates from the P_1 mother (Table 3.1).

The marker data were also analyzed using a heterogeneous residual variance model adapted for binary traits (Xu *et al.*, 1999). This model generates sampling variances of the estimated parameters. The additive genetic variance (V_A) and dominance variance (V_D) estimates for each significant marker are shown in Table 3.2. Marker B20.274 accounts for 96% of the additive genetic variance and 52% of the dominance variance. Markers A06.2461, A09.409, and C13.1385 make a negligible contribution to additive genetic variance, and account for 31%, 5%, and 12% of the dominance variance respectively. A superimposed comparison of likelihood ratio statistics (in the form of LOD scores) generated using CIM and the heterogeneous residual variance method shows a strong correspondence between the two approaches (Figure 3.5).

Table 3.2 Additive genetic and dominance variance estimates for significant markers using the heterogeneous residual variance model of Xu *et al.* (1999).

Marker	V_A	% of V_A	V_D	% of V_D
A06.2461	0.09	2	0.53	31
B20.274	4.56	96	0.88	52
A09.409	0.01	0	0.08	5
C13.1385	0.11	2	0.20	12

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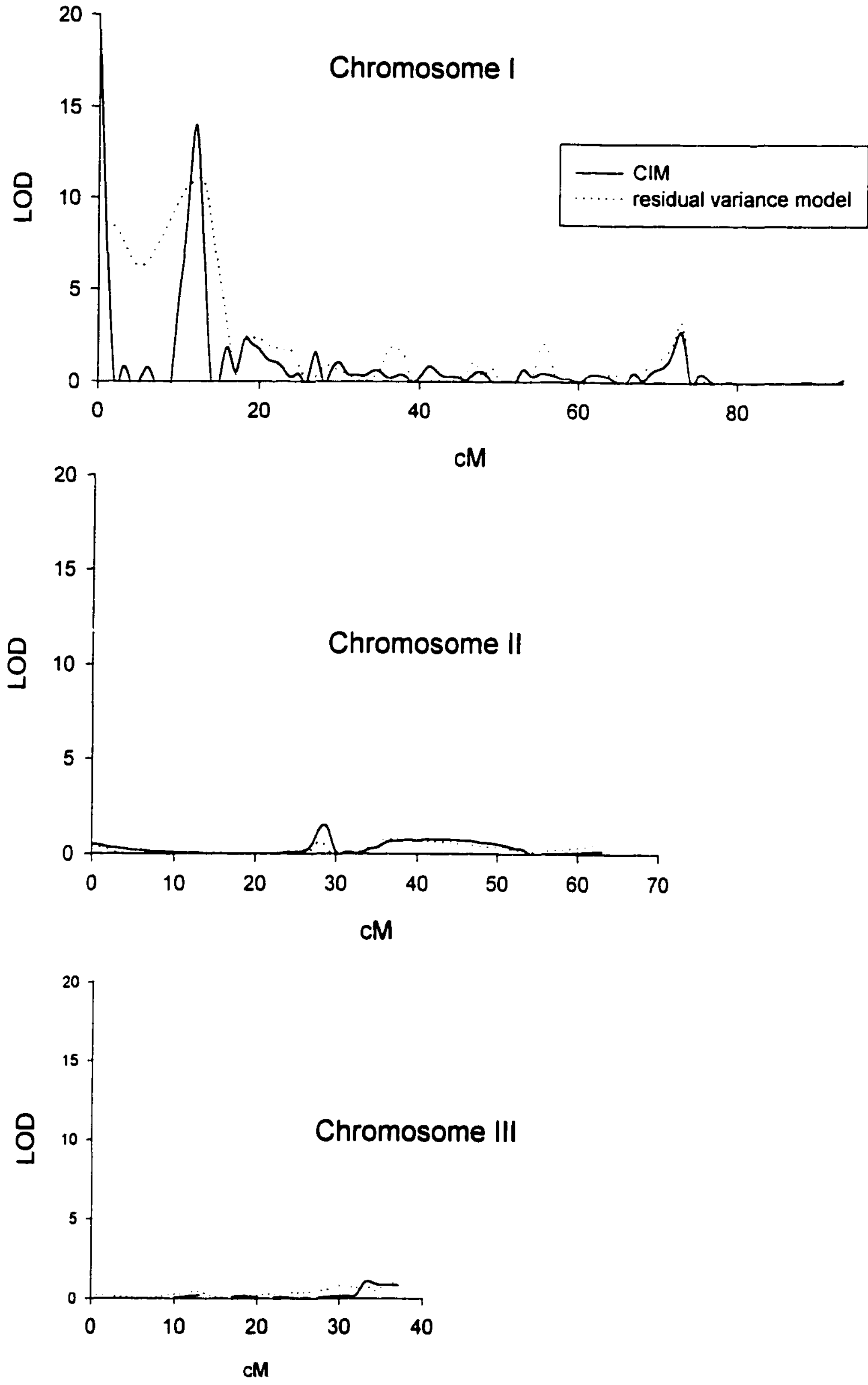

Figure 3.5. A superimposed comparison of likelihood ratio statistics (in the form of LOD scores) generated using CIM and the heterogeneous residual variance model of Xu *et al.* (1999)

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The results of the permutation test for significance of individual LOD scores using a 95% experimentwise error threshold are shown in Figure 3.6. Markers B20.274 and A06.2461 are the only loci with a significant effect on sex. However, the tests performed in each iteration are not independent and therefore the 95% experimentwise error threshold may be too conservative. Thus, it is possible that the peaks associated with marker C04.592 and C13.1385 could in fact be significant (Figure 3.6). The results of the permutation test using a 95% comparisonwise error threshold are shown in Figure 3.7. This test is less stringent in assigning significance, but does not adjust for the large number of individual tests performed.

D. Discussion

My RAPD-SSCP map length of 193 cM for *Ae. triseriatus* is comparable to the 215 cM maps generated from allozymes and morphological markers (Munstermann, 1989; Matthews and Munstermann, 1990). Discrepancies in the lengths of individual chromosomes is likely caused by the latter maps having been constructed prior to the advent of simultaneous multi-locus linkage analysis. The previous maps were assembled by adding the recombination rates observed in two-point crosses in numerous *Ae. triseriatus* families. Such an approach is based on the assumption that recombination rates, like map distances, are additive, which generally holds true only for small recombination distances. Further, they do not account for possible crossover interference that can suppresses recombination.

It is noteworthy that chromosome I is the largest of the three linkage groups. This is corroborated by microscopic measurements of physical chromosome length which also

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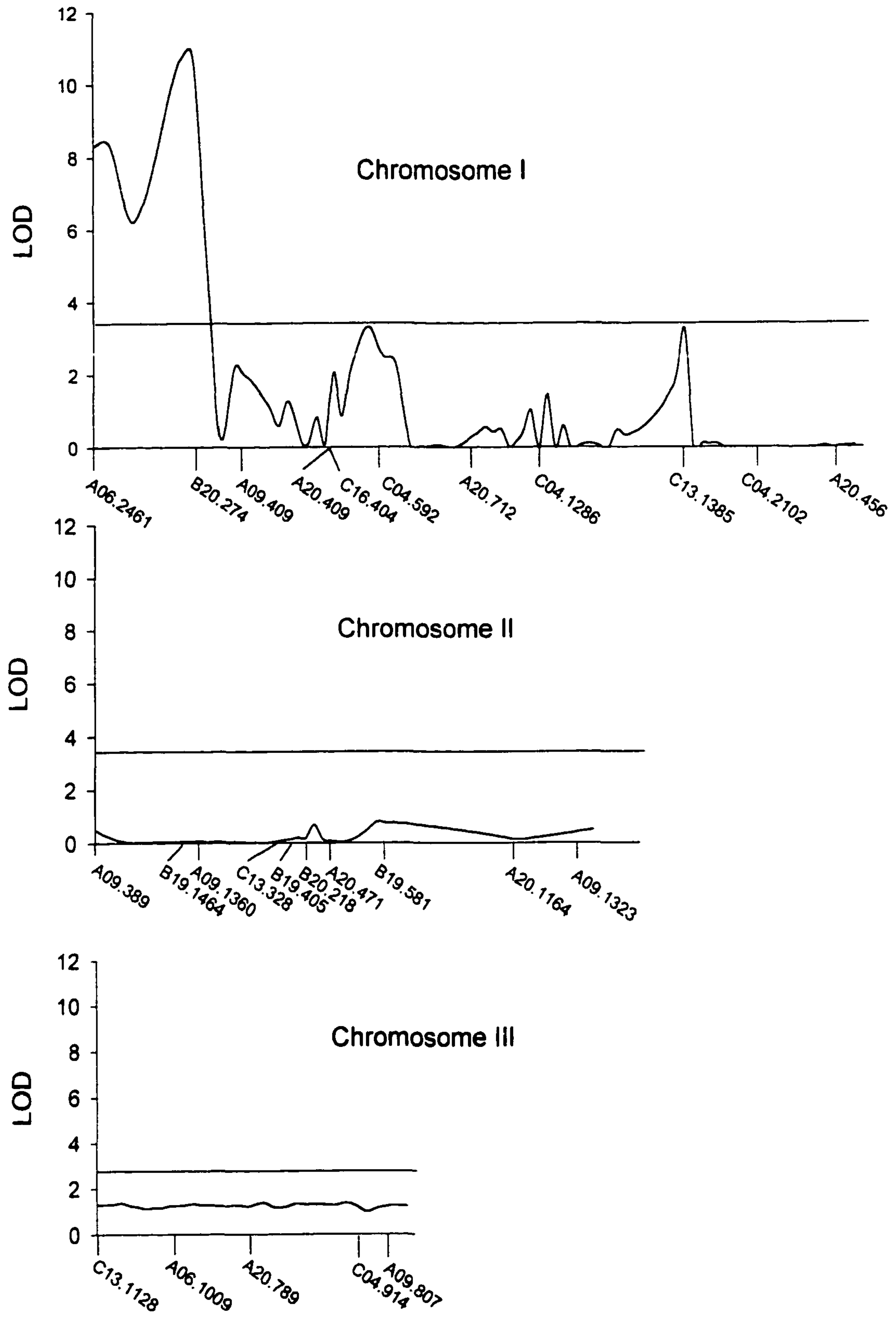

Figure 3.6 Modified LOD scores generated from 1000 iterations of the permutation test of Xu *et al.* (1999) using a 95% experimentwise error threshold of 3.24 (horizontal line).

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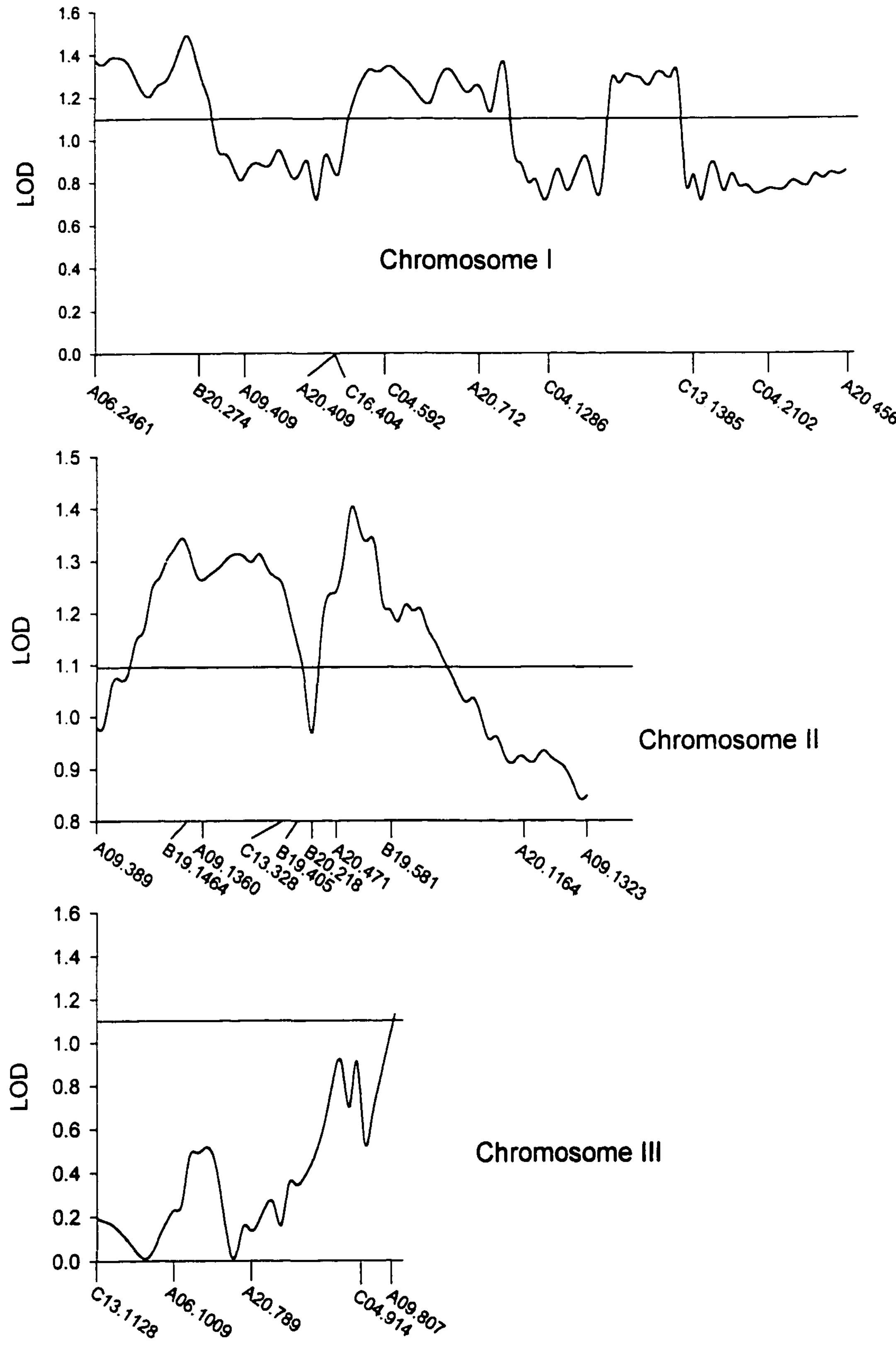

Figure 3.7 Modified LOD scores generated from 1000 iterations of the permutation test of Xu *et al.* (1999) using a 95% comparisonwise error threshold of 1.096 (horizontal line).

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indicated that one chromosome in *Ae. triseriatus* was over 40% longer than the other two (Rao and Rai, 1987a). This is in contrast to RAPD-SSCP linkage studies of *Ae. aegypti* (Antolin *et al.*, 1996) and *Ae. albopictus* (Mutebi *et al.*, 1997) in which chromosome I was the smallest of the three.

With a haploid genome size of 1.52 pg DNA, *Ae. triseriatus* has one of the largest physical genome sizes among mosquitoes, over 90% of which consists of repetitive sequences (Black and Rai, 1988; Rao and Rai,1987b). By comparison, the haploid genome size of *Ae. aegypti* is 0.83 pg DNA, 40% of which is repetitive (Warren and Crampton, 1991), and that of *Ae. albopictus* (Mauritius) is 1.32 pg DNA, 58% repetitive (Black and Rai, 1988). Recombinational map lengths based on RAPD-SSCP markers for *Ae. aegypti* and *Ae. albopictus* are 168 cM (Antolin *et al.*, 1996) and 225 cM (Mutebi *et al.*, 1997) respectively. The absence of a strong relationship between genome size and recombinational length might be explained by differences in the distribution and amount of repetitive DNA within and between these three species. Significant intraspecific variation in genome size has been documented for *Ae. aegypti* (Warren and Crampton, 1991) and *Ae. albopictus* (Black and Rai, 1988). Interspecific differences in the distribution and frequency of chiasmata may also explain this discrepancy (Dev and Rai, 1984; Sherron and Rai, 1984).

Genetic mechanisms causing a male-biased sex ratio have previously been described in *Ae. aegypti* (Hickey and Craig, 1966) and *Cx. pipiens* (Sweeney and Barr, 1978). In each case, a meiotic drive-based genetic model was postulated in which a single dominant distorter gene linked to the male-determining allele *M* rendered that chromosome more likely to participate in fertilization. However, to explain a female-

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biased sex ratio, a reciprocal scenario in which a distorter gene is linked to *m* cannot be invoked as this would affect both sexes equally. Similarly, recessive lethal alleles linked to the sex locus would distort sex ratio in favor of males. In the present study, three regions on chromosome I in addition to the putative sex locus, linked to marker B20.274, show a significant effect on sex. These QTLs, associated with markers A06.2461, A09.409, and C13.1385, map 12 cM, 6 cM, and 59 cM away from B20.274 respectively (Figures 3.2 and 3.3). In light of the significantly distorted sex ratio in this family, these results indicate that several loci in addition to the sex locus may play a role in distorting sex ratio in *Ae. triseriatus.*

A variety of sex determination mechanisms have been documented among the insect families (Hoy, 1994). Among the Diptera, a mechanism whereby femaleness is conditioned by the presence of one or several loci (as the present data suggest) has been described for the house fly, *Musca domestica*. In some strains of *Musca* dominant male-determining loci are found, whereas in other strains, these are overruled by epistatically acting female-determining loci (Hilfiker-Kleiner *et al.*, 1994).

Ultimately, adult sex ratios in mosquitoes can be determined by many variables, from genetic mechanisms acting—sometimes in concert with environmental effects—early in development, to such late-acting factors as differential larval and pupal survival (Clements, 1992). At this time, I cannot draw any conclusions as to the precise mechanism(s) causing the observed sex-ratio distortion in *Ae. triseriatus* other than to state that 1) the female-biased sex ratios observed in this study were not limited to a few families (Figure 3.1), and 2) there appears to be a significant genetic component

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involved. I do not yet know whether this occurs in nature due to the difficulty in rearing families from the field.

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CHAPTER 4

LINKAGE MAPPING OF QUANTITATIVE TRAIT LOCI CONDITIONING TRANSOVARIAL TRANSMISSION OF LA CROSSE VIRUS IN *AEDES TRISERIATUS*

A. Introduction

The eastern treehole mosquito, *Aedes triseriatus*, is the principal vector of La Crosse virus (LAC), an important cause of pediatric arthropod-borne viral (arboviral) encephalitis in the United States (Calisher, 1994). *Ae. triseriatus* females are also capable of transovarially transmitting LAC to their progeny. Given that in the northern part of its range, *Ae. triseriatus* overwinters by facultative egg diapause, and its main rodent hosts do not sustain LAC viremias through the winter, transovarial transmission (TOT) is the principal mechanism by which LAC is maintained transseasonally in this region.

Quantitative trait loci (QTLs) affecting the ability of mosquitoes to transmit pathogens (i.e. vector competence) have been mapped in several important species. These include QTLs affecting the ability of *Aedes aegypti* to transmit Dengue-2 virus (Bosio *et al.*, 1999b), the malaria parasite *Plasmodium gallinaceum* (Severson *et al.*, 1995a), and the filarial worm *Brugia malayi* (Severson *et al.*, 1994), and the ability of *Anopheles gambiae* to transmit *Plasmodium cynomolgi* B (Zheng *et al.*, 1997). However, studies aimed at mapping QTLs conditioning the vertical transmission of pathogens in mosquitoes are lacking. In recent selection experiments, I demonstrated a genetic basis

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to TOT of LAC in laboratory strains of *Ae. triseriatus* (Chapter 2; Graham *et al.*, 1999a), and constructed a linkage map for this species using RAPD-SSCP markers (Chapter 3; Graham *et al.*, 1999b). In the present study I utilize this linkage information to map several QTLs with a significant effect on TOT of LAC in these strains. Identification of loci conditioning this epidemiologically important trait in *Ae. triseriatus* can serve as the basis for future efforts aimed at cloning and characterizing the gene(s) involved.

B. Materials and Methods

***Aedes triseriatus* strains.** The AIDL and Holmen strains of *Ae. triseriatus* originated from eggs collected at different sites near La Crosse, Wisconsin. The AIDL strain originated in 1983 and has been in colony for ~ 30 generations, whereas the Holmen strain, established in 1992, has been through 15 generations. Each colony was maintained at 20-23°C, 75% relative humidity, and a photocycle of 16:8hr L:D with a 60 min crepuscular period at dawn and dusk. Larvae were fed Tetra-Min® fish food. Adults initially were housed in 24 x 24 x 24 in flight cages and consisted of 500 – 1,000 individuals per strain. The Holmen strain was selected for refractoriness to TOT of LAC, while the AIDL strain was selected for permissiveness to TOT of LAC (Chapter 2; Graham *et al.*, 1999a).

Mating scheme. *Ae. triseriatus* is a swarm-breeding mosquito (Wright *et al.* 1966). Copulation and insemination occur only in the presence of numerous males, thereby precluding single pair mating. My attempts at artificially induced copulation were unsuccessful. Therefore paternal RAPD profiles were inferred from the maternal genotype and marker segregation ratios in the offspring. Female mosquitoes mate only

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once (Craig, 1967), thus precluding confounding effects of multiple paternal genotypes among progeny. Experiments aimed at maximizing insemination rates in *Ae. triseriatus* females with a minimum number of males revealed an optimum of 10 to 20 males per 5 females and a minimum cage size of 1 ft x 1 ft x 1 ft (data not shown).

Reciprocal crosses were carried out between the AIDL and Holmen strains. Females were then allowed to bloodfeed on a mouse and removed to individual 1-pint (500 ml) oviposition containers. Eggs were oviposited on strips of moist paper towel. Following oviposition, females and males were frozen for genotyping. For each F_1 family, progeny were reared to adulthood, a subset of 10 males and 5 females were allowed to interbreed, females were given a bloodmeal, eggs were collected for each mother, and parents were frozen for genotyping. A subset of F_2 families were reared in a like manner, except that females were given an infectious bloodmeal and assayed for TOT. The F_2 family used for QTL mapping in this study consisted of 49 individuals (28 female, 21 male) descended from the cross between a P_1 mother from the (TOT-refractory) Holmen strain and a P_1 father from the (TOT-permissive) AIDL strain.

Virus stock. LAC was obtained from the World Health Organization Arbovirus Reference Bank at the Yale Arbovirus Research Unit, New Haven, CT. This virus was originally isolated in 1964 from the brain of a patient with LAC encephalitis (Thompson *et al.*, 1965). The AIDL stock of this virus was passed once in suckling mouse brain and eight times in baby hamster kidney (BHK-21) cells (Chandler, 1995).

Preparation of infectious bloodmeal. LAC was grown in BHK-21 cells in Leibovitz L-15 medium containing 10% fetal bovine serum with penicillin and streptomycin. A cell monolayer (T-75 flask) was incubated with virus at a multiplicity of

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infection of 0.01 at 37°C for 1 hr., after which supernatant was replaced with fresh growth medium. Viral infection of the monolayer was allowed to proceed until producing moderate to severe cytopathic effects. Supernatant then was harvested by centrifugation at 1000 x g for 10 min., and 5 ml added to equal parts of 10% sucrose solution and defibrinated sheep blood (Colorado Serum Co., Denver, CO). Bloodmeal titers ranged between 10^6 and 10^8 Tissue Culture Infectious Dose $(TCID)_{50}$/ml (Karber, 1931) at the beginning of the meal, and decreased by an average of $10^{0.5}$ $TCID_{50}$/ml by the end of blood feeding.

Oral infection of F_2 females. At 5-7 d post-emergence, female F_2 mosquitoes were removed from their cages and placed into several 1-gallon (3.8 L) containers with mesh fabric lids. They were deprived of sugar and water for 24 and 12 hrs, respectively, before blood feeding. The bloodmeal was administered via hanging droplets and artificial membrane feeders (Rutledge *et al.*, 1964). Mosquitoes were allowed to feed for 1 hr. Partially engorged females and those that did not feed were discarded. Bloodfed females were transferred individually to oviposition containers.

LAC does not transovarially infect progeny from the first ovarian cycle (Miller *et al.*, 1979). Therefore, following the first oviposition, a second ovarian cycle was induced with a non-infectious bloodmeal consisting, in a 2:1 proportion, of defibrinated sheep blood and 10% sucrose solution. This bloodmeal was administered separately to each female via hanging droplets. The large number of individual females in pint cartons prohibited the use of artificial membrane feeders. F_3 progeny from the second ovarian cycle were assayed for LAC infection. Within each reciprocal P_1 cross, several candidate F_3 families were assayed.

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Assay of viral infection of mosquitoes. To determine the TOT phenotype of F_2 females, F_3 larvae were reared to the second or third instar, squashed on microscope slides, and assayed for the presence of LAC antigen using anti-LAC antibody/fluorescein isothiocyanate (FITC) conjugate and a fluorescent microscope (Chandler, 1995). Adult females were assayed for disseminated infection by assaying neural tissue in head squashes for viral antigen (Beaty and Thompson, 1978). Distinct fluorescence foci in tissues indicated viral infection. To verify that this technique was sufficiently sensitive to detect the lower levels of virus typical of early instars (Beaty and Thompson, 1976), I assayed transovarially infected mosquitoes by developmental stage. No appreciable difference in infection rate was observed between instars (Chapter 2; Graham *et al.*, 1999a).

DNA isolation and RAPD-PCR. DNA was extracted following the protocol of Bender *et al.* (1983). DNA was resuspended in 1 ml TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0). The majority of the DNA was archived at –80°C; DNA to be used immediately for PCR reactions was stored at 4°C. RAPD-PCR was performed using each of 15 primers (Operon Technologies, Alameda, California). These were A06 (5' GGTCCCTGAC 3"), A09 (5' GGGTAACGCC 3'), A20 (5' GTTGCGATCC 3'), B18 (5' CCACAGCAGT 3'), B19 (5' ACCCCCGAAG 3'), B20 (5' GGACCCTTAC 3'), C01 (5' TTCGAGCCAG 3'), C04 (5' CCGCATCTAC 3'), C13 (5' AAGCCTCGTC 3'), C16 (5' CACACTCCAAG 3'), D2 (5' GGACCCAACC 3'), D3 (5' GTCGCCGTCA 3'), D5 (5' TGAGCGGACA 3'), D8 (5' GTGTGCCCCA 3'), D18 (5' GAGAGCCAAC 3') PCR was performed in 96 well plates. A single large PCR reaction mixture sufficient to perform 100 50 µl reactions was made by mixing 4,350 µl dd H_2O, 500 µl 10X Taq

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buffer (500 mM KCl, 100 mM Tris-HCL pH 9.0), 50 μl of 20 mM dNTPs, and 5000 pm of the primer. This reaction mixture was set under a UV light source (302 nm) for 10 min, after which 10 μl of Taq polymerase were added. The mixture was then dispensed into a 96 well plate. Two μl of template DNA were added to each well, followed by a drop of sterilized mineral oil. Each set of PCR reactions (i.e. one 96 well plate) was checked for contamination by the use of a negative control containing all reagents except template DNA. The plate was then subjected to 45 cycles of (1) 95°C for 1 min (denaturation); (2) 35°C for 1 min (annealing); (3) ramp to 72°C at a rate of 1°C every 8 s; and (4) 72°C for 2 min (extension). A final 72°C extension was carried out for 7 min and the temperature was held at 4°C. Samples were stored at 4°C until electrophoresis. The contents of each well were tested for the presence of amplified products by loading 5 μl from each well onto a 1.5% TBE agarose gel, performing electrophoresis for 15-20 min at 112 V, staining with ethidium bromide, and viewing the gel over a UV transilluminator.

Electrophoresis to detect SSCPs. SSCP analysis and silver staining were performed according to the protocols described by Hiss *et al.* (1994), Antolin *et al.* (1996), and Black and Duteau (1997). Briefly, PCR products (2.5 μl out of 50 μl) were mixed with 4.5 μl of denaturing loading buffer and electrophoresed on large (40 X 50 cm), thin (0.4 mm) glycerol (5%) polyacrylamide (5%, 2% cross-linking) gels. Electrophoresis proceeded at constant current (16 mA) at room temperature for 16 hr (overnight). DNA fragments were detected with silver staining.

Scoring of RAPD-SSCP bands. Amplified markers were scored directly from dried gels by measuring band mobility relative to known size markers (1-kb ladder, BRL

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Laboratories). To estimate sizes of amplified DNA fragments, the reciprocal of fragment size was regressed upon the reciprocal of mobility (Schaffer and Sederoff, 1981). RAPD markers were named by the primer designation followed by a period and the estimated size of the fragment.

The RAPD-SSCP method generates numerous bands, not all of which are repeatable. Spurious bands that appeared in only a few individuals were disregarded. Mendelian segregation was tested in the remainder of RAPD loci by using the JMSLA procedure in JoinMap 2.0 (Stam and van Ooijen, 1995). Loci that failed to meet Mendelian expectations were discarded.

Linkage analysis. F_2 offspring genotypes were analyzed using JoinMap 2.0. Recombination fractions were converted to map units (cM) by the Kosambi mapping function (Kosambi, 1944) using the JMREC procedure of JoinMap 2.0. Initially, a minimal logarithm of odds density (LOD) score of 3.0 was used to group markers. DRAWMAP 1.1 (van Ooijen, 1994) was used to plot a linkage map.

Mapping QTLs affecting TOT of LAC in *Ae. triseriatus*. The computer program QTL cartographer 1.12 (Basten *et al.*, 1997) was used to test for QTLs that affect TOT. Previous experiments have shown that TOT of LAC virus in *Ae. triseriatus* conforms to a single-locus genetic model in which females exhibit either a refractory or permissive phenotype and the filial infection rate (the proportion of a female's progeny that are infected) is not under genetic control (Chapter 2; Graham *et al.*, 1999a). TOT was therefore treated as a binary trait and scored as 0 (refractory) or 1 (permissive). Initially, stepwise linear regression was used to reveal those markers most closely associated with QTLs and to quantify and rank the magnitude of each marker's phenotypic effect.

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Standard (Lander and Botstein, 1989) and composite (Zeng, 1994) interval mapping were then performed. Standard interval mapping (SIM) estimates QTLs at only one location at a time and can give biased results due to the effects that additional QTLs contribute to sampling variance and due to the fact that when two QTLs are linked, their combined effects can yield upwardly biased estimates. Composite interval mapping (CIM) uses a combination of regression and maximum likelihood methods to reduce this bias (Zeng 1994). CIM fits regression coefficients for background markers to account for variance caused by non-target QTLs. The variance from other QTLs is accounted for by including partial regression coefficients from markers in other regions of the genome and thus the effects of other QTLs are not present as residual variance. Following Jansen and Stam (1994), background markers were chosen by stepwise regression using the SRmapqtl procedure in QTL Cartographer. The number of markers used to control for genetic background in CIM was 2.0 and the window size (interval) was 10 cM. Dominance effects were tested by calculating LOD scores for the comparison of the null hypothesis that there are no genetic effects with the alternative that there are dominance effects, but no additive genetic effects (H_2/H_0, Basten *et al.*, 1997).

To confirm the results of CIM, each RAPD-SSCP marker was also analyzed by a contingency χ^2 test to determine if TOT ability is equally distributed among genotypes. The χ^2 analysis was also used to determine if QTLs affecting TOT arose from one or both parents (Table 4.1). Finally, a permutation procedure was used to test the consistency with which the dataset supports a particular QTL (Churchill and Doerge, 1994). Following the initial CIM analysis, the Zmapqtl procedure in QTL Cartographer was used to randomly permute the phenotypic traits and genotypes 100 times and then

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perform CIM in each of the 100 permuted datasets. The program then reported the proportion of permuted replicates that had LOD values larger than the original LOD.

The marker data were also analyzed using a model designed for complex binary traits (Xu *et al.*, 1999). Such traits are derived from the threshold characters of quantitative genetics. Threshold characters have an underlying continuity, termed the liability, with a threshold which imposes a discontinuity on the visible expression (Falconer and MacKay. 1996). Thus, there exists a fixed threshold in the scale of liability, below which an individual expresses one phenotype (e.g. refractoriness to TOT), and above which it expresses another (e.g. permissiveness). The model developed by Xu *et al.* (1999) transforms the liability to a normal distribution using a probit scale, and liability is described using a single linear model with a heterogeneous residual variance. One thousand random iterations of phenotype were performed on a fixed genotype file. In each iteration the program recorded the highest LOD score for each point and the highest LOD score in that iteration. The program generated the 950th highest value for each point (the 95% comparisonwise error rate) and the 950th highest value for each iteration (95% experimentwise error rate). This model also generated estimates of additive genetic variance and dominance variance for each putative QTL.

C. Results

A total of 74 polymorphic RAPD-SSCP markers were identified that segregated in a Mendelian fashion, with an average of 5 markers per primer. At a minimal LOD of 1.4, the JMGRP procedure in JoinMap split the markers into two linkage groups, and at an LOD of 1.7 JoinMap split the markers into three groups. At a LOD of 3.0, the accepted

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threshold for establishing linkage (Basten *et al.*, 1997), the markers were split into eight groups, several of which consisted of just one or two markers.

The largest linkage group was comprised of 35 markers. Interestingly, all markers with a significant χ^2 association with TOT were contained within this linkage group (Figure 4.1B). Therefore I focussed my QTL analysis on only this group. To ascertain which of the three mosquito chromosomes this linkage group corresponds to, I used RAPD primer D03, which yielded six markers on this linkage group, to amplify DNA from a larger *Ae. triseriatus* family for which linkage relationships had already been determined (Chapter 3; Graham *et al.*, 1999b). Two of these six loci (D03.1641 and D03.883) were unequivocally reproduced in the larger family and both mapped to chromosome I (Figure 4.1A). Additionally, marker C04.546 from the larger family (at 57 cM in Figure 4.1A) and marker C04.523 from the present family (at 63 cM in Figure 4.1B) map to roughly corresponding regions on chromosome I and share the same segregation patterns. The difference in fragment size of 23 bp between these two markers falls within the slight variation to be expected when measuring the mobility of the same RAPD fragment across electrophoresis gels (Antolin *et al.*, 1996). Therefore I conclude that the linkage group in question, the one showing a significant association with TOT, corresponds to chromosome I.

Treating TOT as a binary character revealed two putative QTLs on chromosome I with a significant phenotypic effect (Figure 4.2). Markers C01.385 and D03.1505 are associated with an LOD peak (LOD = 2.5) at 57 cM, and a second larger peak (LOD = 4.0) at 72 cM is associated with marker C01.1085. Table 4.1 lists the χ^2 values, P_1 parentage of the TOT-permissive genotype, and permutation test *P* values for each

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Table 4.1. For each marker with a significant association with TOT: the observed and expected numbers of permissive and refractory F_2 females for each marker genotype as a function of P_1 parentage, χ^2 value and *P* value from the permutation test. (xs = excess, def = deficiency)

marker		RAPD-SSCP marker from P_1 mother		RAPD-SSCP marker from P_1 father		χ^2 value[a]	*P* value
		permissive	refractory	permissive	refractory		
C01.385	obs.	6 (def)	8 (xs)	13 (xs)	1 (def)	8.02***	0.01
	exp.	9.5	4.5	9.5	4.5		
D03.1505	obs.	17 (xs)	3 (def)	2 (def)	6 (xs)	9.43***	< 0.0001
	exp.	13.6	6.4	5.4	2.6		
D02.1172	obs.	7 (def)	8 (xs)	12 (xs)	1 (def)	6.65**	0.02
	exp.	10.2	4.8	8.8	4.2		
D02.953	obs.	7 (def)	8 (xs)	12 (xs)	1 (def)	6.65**	0.02
	exp.	10.2	4.8	8.8	4.2		
B20.694	obs.	6 (def)	7 (xs)	12 (xs)	1 (def)	6.50**	0.02
	exp.	9.0	4.0	9.0	4.0		
B20.392	obs.	2 (def)	6 (xs)	16 (xs)	3 (def)	8.88***	0.04
	exp.	5.3	2.7	12.7	6.3		
B19.745	obs.	12 (xs)	2 (def)	7 (def)	7 (xs)	4.09*	0.05
	exp.	9.5	4.5	9.5	4.5		
D03.1641	obs.	3 (def)	6 (xs)	16 (xs)	3 (def)	7.25**	0.04
	exp.	6.1	2.9	12.9	6.1		
D03.883	obs.	3 (def)	6 (xs)	16 (xs)	3 (def)	7.25**	0.04
	exp.	6.1	2.9	12.9	6.1		
C01.1085	obs.	2 (def)	6 (xs)	17 (xs)	3 (def)	9.43***	0.03
	exp.	5.4	2.6	13.6	6.4		

[a] (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$),

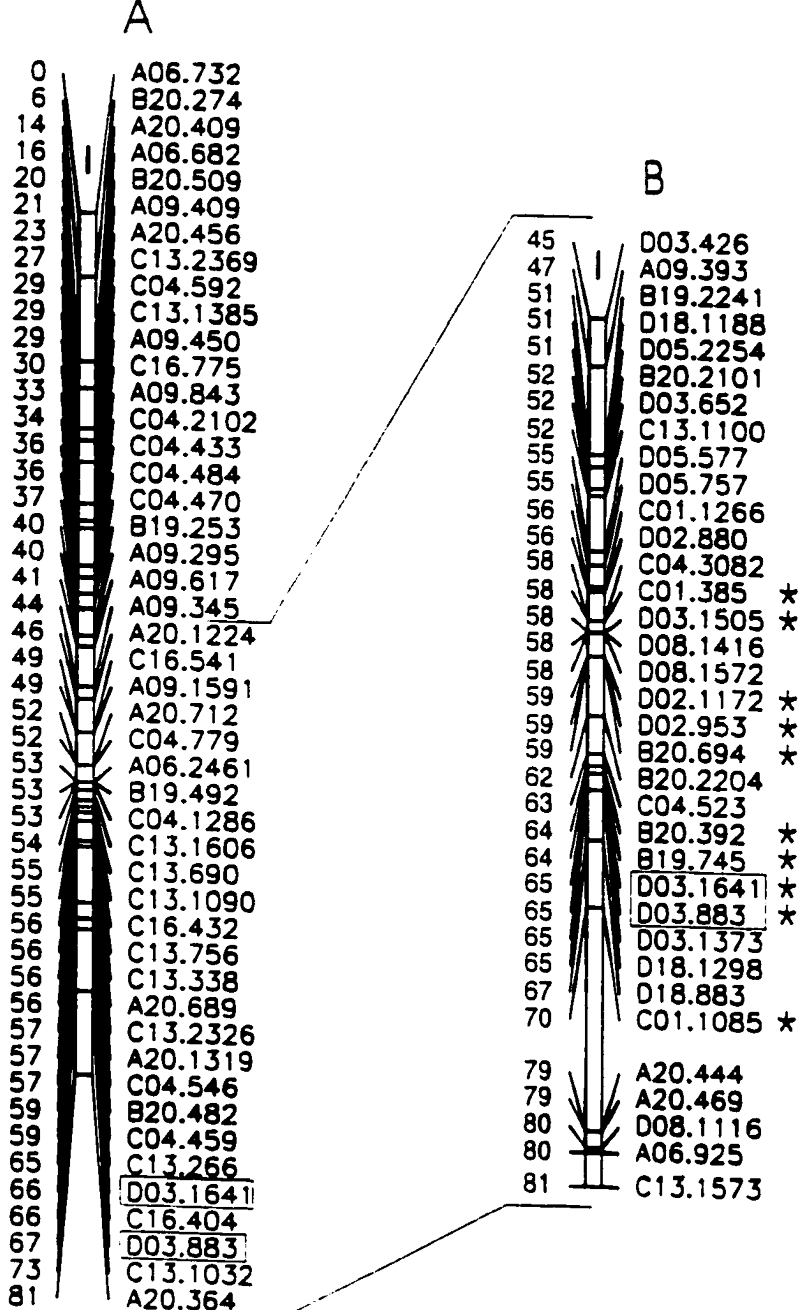

Figure 4.1 A. RAPD-SSCP linkage map of chromosome I in *Ae. triseriatus* based on segregation of markers in a family of 146 individuals (Chapter 3; Graham *et al.*, 1999b). **B.** RAPD-SSCP linkage map of a segment of chromosome I in the present study. Map units on the left of each linkage group are in centiMorgans. Each marker is designated by the name of the RAPD primer, followed by a period, then the size in bp of the amplified fragment. Asterisks indicate markers with a significant association with TOT, according to the permutation tests of Churchill and Doerge (1994). Markers common to both families are indicated in boxes.

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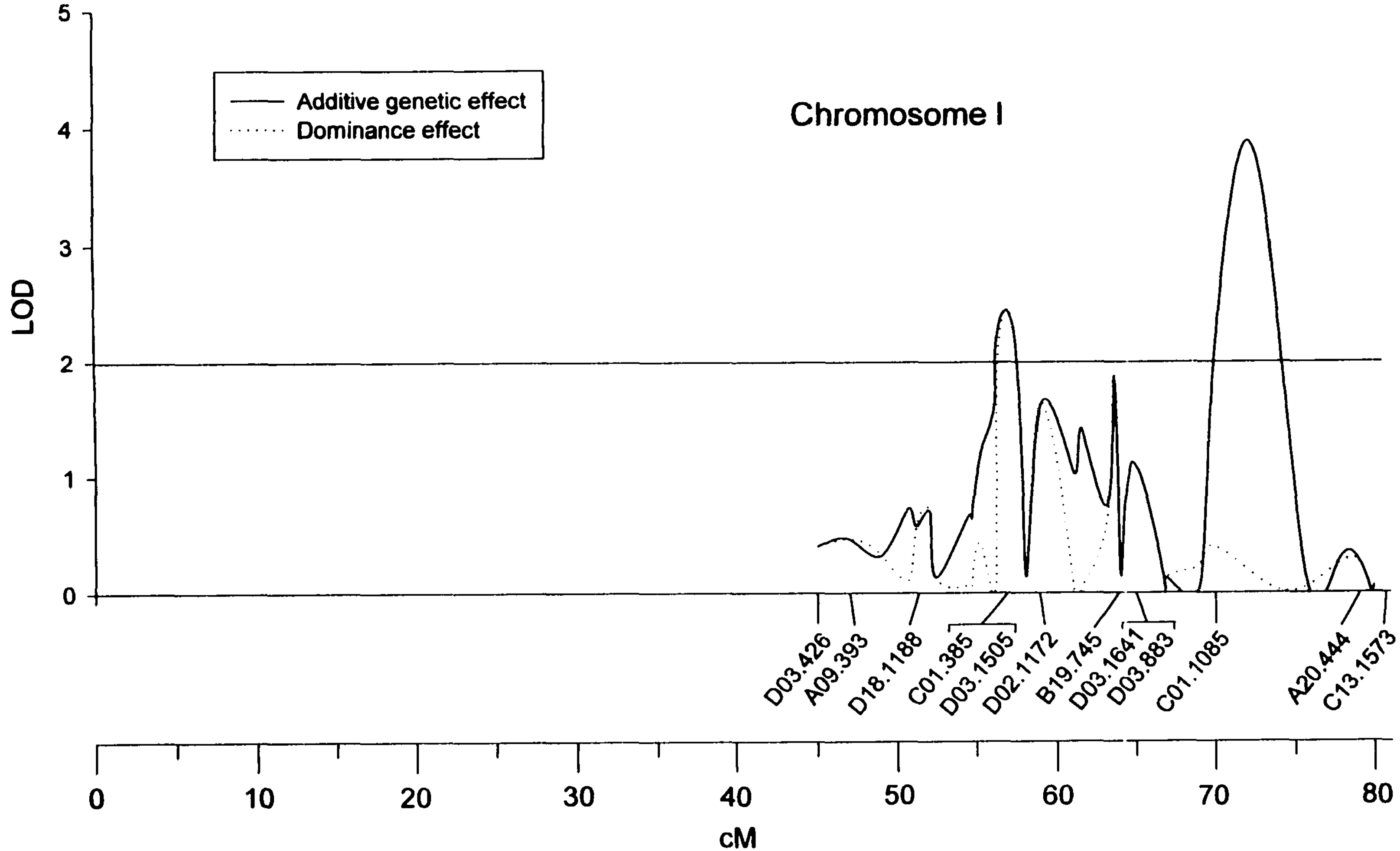

Figure 4.2 Plots of LOD scores for TOT on *Ae. triseriatus* chromosome I. LOD values were estimated using composite interval mapping. The X axis is proportional to the recombinational distance of chromosome I in Figure 4.1A (Graham *et al.*, 1999b). Locations of selected markers are shown relative to their positions on the linkage map (Figure 4.1B).

marker showing a significant association with TOT ability. For eight of the ten markers, the dominant allele inherited from the P_1 mother is associated with an excess in refractory F_2 females, while the recessive allele inherited from the P_1 father is associated with an excess of permissive F_2 females.

The marker data were also analyzed using a heterogeneous residual variance model adapted for binary traits (Xu *et al.*, 1999). Using a 95% experimentwise error threshold, only one locus, corresponding to marker C01.1085, was significant (Figure 4.3A). This corresponds with the larger of the two LOD peaks obtained with CIM (Figure 4.2). Using the less conservative 95% comparisonwise error threshold, seven peaks were significant (Figure 4.3B). The markers corresponding to these peaks are listed in Table 4.2, along with their relative contributions to additive genetic variance and dominance variance. The comparisonwise test does not adjust for the large number of tests performed and is probably too liberal in assigning significance. The actual number of significant loci is probably most accurately revealed by CIM.

Table 4.2. Relative contributions to overall additive genetic variance and dominance variance of loci that were significant using the 95% comparisonwise error rate of Xu *et al.* (1999).

marker	V_A	% of V_A	V_D	% of V_D
D18.1188	.069	1	.175	2
D05.757	2.839	25	2.235	31
C01.1266	.091	1	.155	2
D03.1505	.019	0	.001	0
B20.2204	3.155	28	1.799	25
C04.523	4.449	40	1.400	20
B20.392	.226	2	.051	1
D18.883	.258	2	.662	9
C01.1085	.079	1	.432	6
C13.1573	.037	0	.197	3

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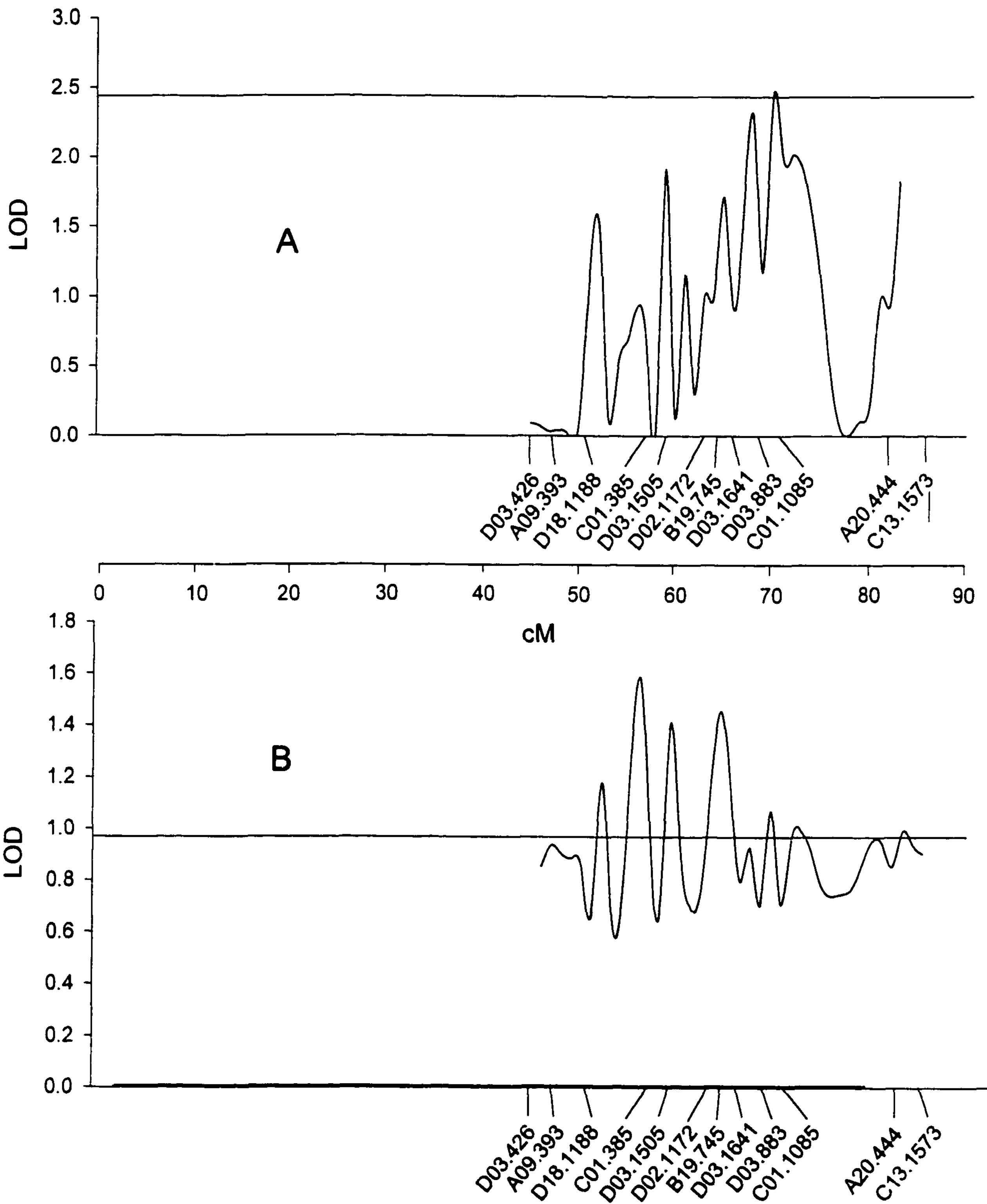

Figure 4.3. Modified LOD scores generated from 1000 iterations of the permutation test of Xu *et al.* (1999) using A) a 95% experimentwise error threshold of 2.419 and B) a comparisonwise error threshold of 0.967 (horizontal lines). The centiMorgan scale applies to both graphs.

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D. Discussion

The P_1 parents used in this study originated from AIDL and Holmen strains of *Ae. triseriatus* that had been subjected to a number of generations of selection (Chapter 2; Graham *et al.*, 1999a). This resulted in a decrease in overall fitness and significantly reduced family sizes. The family size used in the present study (49 individuals from three gonotrophic cycles) is below the ideal for linkage mapping (Maliepaard *et al.*, 1997). This largely explains why, at a minimal LOD of 3.0 the JMGRP procedure of JoinMap split the markers into eight linkage groups. It is unlikely that the addition of more markers would cause them to coalesce into three groups, the correct haploid number of chromosomes for a mosquito genome.

The small sample size of 28 females used for QTL mapping may have contributed to the large number of markers with a significant association with TOT (Table 4.1). Also, these markers are all located within a 12 cM span that maps toward the telomere of chromosome I (Figure 4.1A) The reduced crossing over typically observed in telomeric regions probably played a role in "locking" so many markers into a significant association with the QTLs.

One limitation to using molecular marker approaches for QTL detection is that only those QTLs with a sufficiently large phenotypic effect to be detected statistically will be counted (Tanksley, 1993). This limitation is exacerbated as sample size decreases. That I detected two QTLs in such a small segregating population is an indication that these QTLs have a significant effect on TOT, despite relatively low LOD scores of 2.5 and 4.0.

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This is consistent with previous experiments which showed a strong response to selection on TOT in *Ae. triseriatus* (Chapter 2; Graham *et al.*,1999a).

As revealed by CIM, the two significant LOD peaks shown in Figure 4.2 differ considerably in the relative contributions of additive and dominance effects. The smaller peak at 57 cM has a very pronounced dominance effect, equal in magnitude to the additive genetic effect. In contrast, the larger peak at 72 cM is comprised almost entirely of additive genetic effect. This suggests that the QTLs associated with each peak are independent and come from opposite sides of the P_1 cross. This is corroborated by the P_1 parentage of markers in Table 4.1. For marker D03.1505, associated with the smaller LOD peak, the dominant allele associated with an excess in refractory F_2 females comes from the P_1 father. For marker C01.1085, associated with the larger LOD peak, the dominant allele associated with an excess in refractory F_2 females is inherited from the P_1 mother.

However, the estimates of the relative contribution to V_A and V_D for these two loci using the heterogeneous residual variance model of Xu *et al.* (1999) (Table 4.2) are not consistent with the peaks generated by CIM. Using the model of Xu *et al.* (1999), D05.1505 makes only negligible contributions to both V_A and V_D, while C01.1085 contributes more to V_D (6%) than it does to V_A (1%): the opposite of the results using CIM (Figure 4.2 and Table 4.2). The LOD peaks obtained with CIM show a reasonable correspondence with those generated with the model of Xu *et al.* (1999) using the 95% experimentwise error threshold. Both methods detected LOD peaks at markers C01.1085 and D03.1505, although D03.1505 was not significant in the experimentwise test (Figure

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4.2 and Figure 4.3A). In light of the conservative nature of the experimentwise test, it is possible that marker D03.1505 was a false negative.

In previous experiments, the response of *Ae. triseriatus* to selection on TOT was described as being consistent with a single-locus model in which permissiveness to TOT is conditioned by a dominant allele (Chapter 2; Graham *et al.*, 1999a). However, the possible effect of a second locus was noted in these experiments, owing to a small proportion of permissive females that descended from refractory mothers. The present study provides additional support to the notion that TOT might be conditioned by two separate QTLs.

However, of all hypothetical two-locus scenarios considered, none predicts an expected phenotype ratio consistent with the data. Unfortunately, even if a genetic model that adequately explained these results were deduced, and even if it ultimately enjoyed statistical validation with a larger data set, its explanatory power in the present study would be significantly weakened by the small sample size. QTL detection using an F_2 analysis is normally carried out with segregating populations of 100 or more individuals (Tanksley, 1993). Thus, with only 28 individuals, any recombination between markers and linked QTLs greatly magnifies the disruptive effect on marker-trait association. Although the marker linkage relationships in this study are corroborated by results from previous mapping experiments (Chapter 3; Graham *et al.*, 1999b), it is premature to draw any firm conclusions from the QTL analysis.

The inbreeding depression observed in my mosquito families is not uncommon in cases where strains have undergone repeated selection. In future experiments, marker assisted selection (MAS) could be used to circumvent this. In MAS, loci that are tightly

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linked to QTLs are characterized in order to choose breeding material in a selection program. Genotypes at QTLs are inferred by the genotypes of flanking markers (Lande and Thompson, 1990; Gimelfarb and Lande, 1994). In this way, only small regions of the genome are exposed to intense selection, thereby mitigating many of the deleterious effects of inbreeding. Larger families could then be generated through selection, while at the same time allowing finer scale QTL mapping.

MAS would also allow the production of mosquitoes that are homozygous at QTLs that condition TOT. With a uniform genetic background in the vector, one could then test the effect of different environmental parameters on TOT as well as genetic variation in different LAC virus isolates. This would also allow one to test whether the genetic penetrance of TOT-refractory QTLs is sufficiently high to justify future attempts at cloning these genes. Finally, MAS would allow the introgression of desired QTL genotypes into desired genetic backgrounds, thereby incorporating desired TOT-refractory traits into stocks designed for release in the wild in an attempt to reduce the incidence of TOT in natural populations.

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CHAPTER 5

SUMMARY AND PERSPECTIVE

These experiments reveal several important aspects regarding the genetics of TOT of LAC in *Ae. triseriatus*. Perhaps most important is the confirmation that there is a heritable basis to this trait in the vector, as evidenced from the positive response to selection documented in chapter 2. The observation of a response to selection on TOT rate, but not on FI rate, provided additional insight into the putative mechanism(s) controlling the dissemination of virus to the oocytes. Namely, TOT has a genetic basis, while FI rate is influenced by environmental factors not under genetic control. The overall response to selection was largely consistent with a single-locus genetic model in which permissiveness to TOT is conditioned by a dominant allele. However, the selection data indicated the possible role of a second locus of lesser effect.

It was postulated that the gene product(s) of this locus (or loci) possibly acted as a membrane-bound or soluble viral receptor associated with one of the anatomical barriers to viral dissemination within the mosquito (Chapter 2; Graham et al., 1999a). Alternatively, the locus in question could be associated with the mosquito's ability to mount an immune response against viral invasion. For example, the locus could condition the ability of virus-infected cells to undergo programmed cell death (apoptosis).

Our RAPD-SSCP map length of 193 cM for *Ae. triseriatus* is comparable to the 215 cM maps generated by Munstermann (1989) and Matthews and Munstermann (1990) using allozymes and morphological markers. As mentioned earlier, this size discrepancy

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is probably the result of recent improvements in mapping technique: the latter maps were constructed prior to the advent of simultaneous multi-locus linkage analysis. The recombinational map length of *Ae. triseriatus* is comparable to that of *Ae. aegypti* and *Ae. albopictus*, the two other mosquito species for which RAPD-SSCP linkage maps have been constructed. However, the physical genome size of *Ae. triseriatus* is considerably larger than that of these other two species, suggesting that little if any recombination occurs in this additional DNA.

QTLs affecting female-biased sex ratios in *Ae. triseriatus* were placed on this linkage map. This revealed several loci, in addition to the sex locus itself, that condition this phenotype. Although female-biased sex ratios in culicine mosquitoes have been described in the context of environmental stress, this is the first documentation of a genetic component associated with this phenomenon.

Mapping QTLs affecting TOT of LAC virus in *Ae. triseriatus* confirmed that there were indeed two separate loci affecting this trait, one with a significant dominance effect, the other with mostly additive genetic effect. Unfortunately, the small sample size in this study hampered linkage map construction. At an LOD of 3.0, the significance threshold for establishing linkage, the grouping subroutine in JoinMap split the markers into eight groups, considerably more than the ideal of three. It was fortuitous that all RAPD-SSCP markers with a significant effect on TOT were grouped together, allowing us to focus our QTL analysis on just this group. It was also fortunate that we were able to find two markers in this group that mapped in common with the larger AIDL family on which our *Ae. triseriatus* linkage map is based. Normally, RAPD markers are difficult to reproduce between families. By anchoring this marker group to the linkage map we were able to

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determine that both QTLs affecting TOT map to within 15 cM of each other on chromosome I. In this case (albeit a rare one given the low cross-family reproducibility of RAPD markers), combining linkage maps proved especially useful.

Certainly, this cross-family linkage alignment could benefit from additional support. Markers generated from targeted PCR could provide this. One promising technique, a variation on the sequence-tagged site (STS) approach, is based on designing 20mer targeted primers from cloned and sequenced RAPD fragments. When template DNA is reamplified with the larger targeted primers, the resulting banding patterns tend to be considerably more informative than with RAPDs: the incidence of null alleles is significantly reduced and band presence-absence is often replaced by codominance. Bosio and colleagues (1999c) have used this approach (STAR; SSCP analysis of Targeted and Amplified RAPD fragments) successfully to anchor chromosomes in two *Ae. aegypti* families.

Additionally, the STAR method can be used essentially as an STS to enhance the resolution of QTL mapping. STAR primers that flank a QTL can be used as probes which would then be used to locate the QTL within ordered cloned fragments of the genome. In theory, one or more of the clones between STAR primer sites would contain the gene(s) of interest. Sequencing these clones and testing for open reading frames would greatly narrow the pool of genes in which to locate a QTL. Thus, by combining the STAR method with a large family size, the resolution and explanatory power of any subsequent QTL analysis would be greatly enhanced.

As mentioned previously, one potential use of QTL mapping incorporates the use of MAS to develop highly TOT-refractory strains for release into natural populations to

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reduce the incidence of TOT. Since the results of these studies indicate that there is more than one gene involved, this would be more difficult. Furthermore, because of environmental and stochastic effects, even a highly TOT-refractory mosquito may transmit virus to her progeny. However, the TOT competency of a population need not be reduced to zero for this strategy to be effective. In theory, reduction of TOT below a threshold could break the cycle of transseasonal maintenance. Reaching this threshold would probably take longer than if vector competence were the target, since virus would still be able to cycle in nature through horizontal transmission. Such an approach would most likely require repeated mass release of selected refractory strains.

An ultimate goal of QTL mapping is to clone and characterize the genes that condition TOT. Since the basis of QTL mapping is the linkage map, linkage maps must be reconciled with a physical map to allow the cloning of genes. Unfortunately (and this is also germane to STS-related cloning and sequencing), the relationship between genetic and physical distance in the *Ae. triseriatus* genome is roughly 8 Mbp/cM (Chapter 3; Graham *et al.*, 1999b; Black and Rai, 1988), rendering map-based cloning infeasible at present. However, as genomic technology steadily advances, and as research on vector genetics continues to expand our knowledge base in this area, it is not unreasonable to assume that in the near future many of today's technique-based obstacles to genetic manipulation will be overcome.

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