

DISSERTATION

BLUETONGUE VIRUS AND *CULICOIDES VARIIPENNIS*: MOLECULAR
EPIDEMIOLOGY, TRANSEASONALITY, AND TAXONOMY

Submitted by

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ABSTRACT OF DISSERTATION

BLUETONGUE VIRUS AND *CULICOIDES VARIIPENNIS*: MOLECULAR EPIDEMIOLOGY, TRANSEASONALITY, AND TAXONOMY

Bluetongue virus (BTV) remains a serious threat to the livestock industry in the United States, causing substantial direct and indirect costs to sheep and cattle producers. As with most arthropod-borne viruses, BTV epidemiology is not well understood. In order to design cost-effective and efficient control protocols, the vector-virus-host disease cycle must be thoroughly investigated. The biting midge, *Culicoides variipennis*, is the primary vector of BTV in the U.S. In Colorado, the winter weather is severe enough to break the BTV transmission cycle every year by killing the insect vectors in the area. The mechanism of observed BTV persistence, or transeasoonality, in these geographical areas despite this cycle interruption year after year is unknown. Studies were conducted to investigate the possibility that vertical transmission in the insect vector acts as the transeasoonality or overwintering mechanism of BTV in temperate regions such as Colorado. In addition, vertebrate

host preference and temporal feeding activities of *Culicoides* in Colorado were studied. Finally, new techniques were developed and characterized for differentiating *Culicoides* species as adults, pupae, and larvae and for detecting BTV analyte in specimens.

Intensive field studies were conducted in Colorado to investigate the transeasonality of BTV in endemic areas. These studies included vertebrate sentinel animal and herd animal surveillance, and extensive larvae and adult *Culicoides* vector collections from four farm sites. Nucleic acid hybridization, antigen detection enzyme immunoassays, and virus isolation techniques were used to investigate the putative role of the arthropod vector in BTV overwintering in temperate regions. Approximately 62,050 larvae were collected over a two year period. Eighty-seven larval pools tested positive by at least one assay; thirteen pools tested positive by two assays. The ELISA was a satisfactory screening test for BTV; virus isolation, however, had a low sensitivity for detecting BTV in these larval samples. At least one of the sentinel calves seroconverted during the study period showing that BTV transmission was occurring in the study areas. Minimum field infection rates (MFIR's) in *Culicoides* at the Ausman site ranged from 0% to 0.8% in 1991 and 0% to 0.4% in 1992. At the Fields site, MFIR's ranged from 0% to 0.5% in 1991, and 0% to 0.3% in 1992. MFIR's at

the Howard farm ranged from 0% to 0.2% in 1992. Subsequent chi-square analysis showed a significant difference in the study site monthly MFIR's during June 1991 and significant differences in the total MFIR's between the Ausman and Fields site in 1991 and 1992. A time-line analysis of the study data showed a BTV-positive larvae peak in June, a BTV-positive adult *C. variipennis* peak in July, and BTV seroconversion of sentinel and herd animals in September and October. The implications of these results for the overwintering hypothesis are discussed.

Prospective studies were conducted to investigate host preferences and temporal and seasonal feeding activities of *C. variipennis* in Colorado. These are major determinants of vectoral capacity of *C. variipennis* for BTV. Populations of insects attracted to sentinel animals at two farm sites were sampled to investigate host attraction rates, seasonal activity periods, site differences and BTV-field infection rates. During the study, approximately 11,000 insects, including *C. variipennis*, were collected. Blood feeding of *C. variipennis* initially peaked in June at the beginning of the study and again in August at both farm sites. The Fields site was monitored an extra month and high numbers of *C. variipennis* were recovered in early September. The calf consistently attracted greater numbers of *Culicoides* species insects than the sheep at both farm sites, except for *C.*

crepuscularis, which preferred the sheep to the calf. MFIR's of *C. variipennis* collected during the study were 4.3% at the Fields site and 10.5% at the Ausman site, which was a significant difference ($p < 0.001$). MFIR's of *C. crepuscularis* were 6.5% at the Fields site and 12.2% at the Ausman site, which was not a significant difference ($p > 0.1$).

Other species of *Culicoides* can transmit BTV world-wide and may play an as yet unknown role in BTV transmission in the U.S. The accurate identification of these vectors to the species level has proven to be difficult, relying almost exclusively on adult morphological characteristics. Accurate species identification is often needed early in the vector life cycle to ensure effective control strategies. To address these problems, PCR amplification of tRNA gene sequences using consensus primers was investigated as a taxonomic tool to differentiate *Culicoides* species in different life stages. This technique was capable of differentiating four species of *Culicoides* commonly found in Colorado. This differentiation could be observed regardless of the life stage of the midge (i.e., adult, pupal, or larval). The technique was also able to discriminate between heterogeneous pools versus homogeneous pools of *Culicoides* species.

A new molecular approach for BTV diagnosis was developed by coupling a reverse transcription-polymerase

chain reaction (RT-PCR) with a reversible target capture (RTC) hybridization assay. This RTC-RT-PCR assay was used to detect analyte in BTV-infected cell cultures and BTV-infected *Culicoides variipennis*. Synthetic oligonucleotide primers were used as both poly-A tailed capture probes and as RT-PCR primers to amplify the BTV analyte to easily detectable levels. By using magnetic-bead separation, the assay bypassed the need for stringent RNA purification techniques, proteinase K treatment and other procedures necessary for the release of this cell-associated virus. This assay showed promise in detecting BTV in clinical specimens such as blood and tissue. The RTC step eliminated agents or factors that inhibit PCR.

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I. LITERATURE REVIEW

A. Historical Introduction to Bluetongue Virus

Arthropod-borne viruses (arboviruses) comprise a large and diverse group of both pathogenic and non-pathogenic viruses transmitted to vertebrates by arthropod vectors. Bluetongue virus (BTV), transmitted principally by biting gnats of the genus *Culicoides*, causes bluetongue (BT) disease in wild and domestic ruminants. BTV is widely distributed and has been isolated in Africa, Middle East, South-East Asia, Mediterranean, North America, and Australia (Gorman, 1990).

BTV apparently originated in South Africa, where it was first described by Hutcheon (1902) and Spruell (1905). The disease was originally called "Malarial Catarrhal Fever of Sheep". The first recorded outbreak of BT outside of Africa occurred on Cyprus in 1949 (Gambles, 1949). Subsequently, the virus has spread panzootically. Even though BTV is distributed through most of Africa, BT disease is rare, occurring usually where sheep industries have introduced European fine-wool and mutton breeds. Native sheep have a high level of innate resistance. Serological studies have documented the presence of BTV in Sudan (Abu Elzein, 1986,

Eisa et al., 1983), Sultanate of Oman (Taylor et al., 1991), Nigeria (Moore, 1974a,b; Exeifeka et al., 1983), Iran (Afshar and Kayvanfar, 1974), Egypt (Barsoum, 1992), Mexico (Stott et al., 1989), Central America (Homan et al., 1990), Colombia (Homan et al., 1985a,b), Peru (Rosadio et al., 1984), Chile (Tamayo et al., 1985), Ecuador (Lopez et al., 1985), India (Jain et al., 1986; Dubey et al., 1988), Indonesia (Sendow et al., 1986; Miura et al., 1982), China (Shi et al., 1992), Malaysia (Hassan, 1992), Cameroon (Ekue et al., 1985a,b), Malawi (Haresnape et al., 1988), Zimbabwe (Jorgensen et al., 1989), Australia (Della-Porta et al., 1983), New Guinea (Van Kammen and Cybinski, 1981) and British Columbia (Sellers and Maarouf, 1991).

In the U.S., BT was first described in Texas in 1948. In 1952, Hardy and Price described a disease called sore muzzle of sheep with clinical manifestations similar to BT disease described by Hutcheon and Spruell. McKercher et al. (1953) were the first to isolate and identify BTV in California in 1952.

B. Economic Importance of Bluetongue Virus

BTV is an economically significant veterinary pathogen. Direct and indirect economic losses in the international market have been estimated to be \$100-125 million yearly (Holbrook, 1988, Walton and Osburn, 1992). In a 1979 epizootic in Mississippi and surrounding states, direct

losses were estimated to be over \$6,000,000 in Mississippi alone with another \$6,000,000 in losses in Alabama, Arkansas, Tennessee, and Louisiana (Metcalf et al., 1982). The prevalence rate in the U.S. of BTV-infected cattle at this time was estimated to be 18.2% (Metcalf et al., 1981). BTV-seropositive cattle at this time had been reported from all of the states with the exception of Alaska and Rhode Island (Callis, 1985). The lowest incidence was in New England and the highest incidence was in the South and Southwest (Metcalf et al., 1981). World-wide losses are estimated at 3 billion dollars annually (Bath, 1989). Indirect economic losses due to weight and wool losses (including poor or slow growth of wool), and lower breeding efficiency, far exceed direct animal losses (Erasmus, 1975a). In addition, import restrictions on livestock and germplasm from BTV-endemic zones into BT-free zones such as those of the European Common Market also cause significant losses. After isolation of BTV from insects for the first time in Australia in 1977, thirty-two countries immediately imposed bans on livestock imports from Australia, even in the absence of clinical disease in the national sheep flock (Doyle, 1992). In South Africa, BT disease is considered the most important limit to animal productivity (Schultz and Grieder, 1987).

C. Bluetongue Virus.

1. Taxonomic relationships

BTV is the prototype virus of the genus *Orbivirus* in the family *Reoviridae* (Roy 1989). The orbiviruses are classified into 13 serological groups that include: African horsesickness, bluetongue, Changuinoloa, Corriparta, epizootic hemorrhagic disease, equine encephalosis, Eubenangee, Kemerovo, Palyam, Umatilla, Warrego, and Wallal viruses (Gorman et al., 1985). Colorado tick fever virus, formerly a member of the *Orbivirus* genus, has recently been designated as the prototype member of the genus *Coltivirus* (Calisher, 1994). The genome of BTV is composed of ten double-stranded ribonucleic acid (RNA) segments enclosed in an icosahedral capsid with a total molecular weight of 18×10^6 daltons. Each segment codes for one unique polypeptide product, and most have been cloned and sequenced. There are no secondary cleavages of the primary gene products. The group-reactive antigenic determinants or epitopes are found mainly on an inner core protein, while the type-specific antigens are located on a surface protein. Twenty-four immunologic serotypes exist, of which five (types 2, 10, 11, 13, and 17) are known to exist in the U.S. (Gorman, 1992). A proposed 25th serotype, designated I-134, has recently been described and characterized (Davies et al., 1992). Originally isolated in Kenya in 1965, I-134 has been

subsequently isolated from severe clinical outbreaks of BTV in wool sheep seven times in Kenya. The different serotypes of BTV vary widely in their geographic distribution and host pathogenicity. Bluetongue virus serotype 3 (BTV-3), originating in South Africa, was found to be extremely virulent when inoculated into British sheep (Jeggo et al., 1986b). First isolated in South Africa by Theiler in 1905, BTV-4 seems to be the most widespread of all the existing BTV serotypes and has been isolated outside of Africa in Turkey and Cyprus. BTV-20, originally called CSIRO 19, was identified from insects collected in the Northern Territories of Australia but appears to be of low pathogenicity to sheep (Uren and Squire, 1982). BTV-20 was isolated from a pool of 214 *Culicoides* of mixed species (Standfast et al., 1984). BTV-20 and BTV-4 are closely related serologically (Della-Porta et al., 1981a). BTV-4 was originally serially passaged in sheep and then used to immunize sheep in the early 1900's (Theiler, 1908). Although a poor vaccine, it was used for more than 20 years in South Africa (Gorman et al., 1983). BTV-21, originally called CSIRO 154, was originally isolated from cattle blood in Australia and seems to have the same pathogenicity for sheep as BTV-20 (Uren and St. George, 1985). BTV-21 is related to BTV-6 (Della-Porta et al., 1981b). Australian serotypes BTV-1 (CSIRO 156), BTV-3, BTV-9, BTV-15, BTV-16,

and BTV-23 are reported to cause only mild disease in sheep in Australia (Flanagan et al., 1993; Gard et al., 1987). BTV-1, BTV-3, BTV-4, BTV-6, BTV-8, BTV-12, BTV-14, and BTV-17, circulate in the Caribbean region and Central America (Gumm et al., 1984; Hoff et al., 1992). Serotypes BTV-7, BTV-18, and BTV-19 have been isolated in South Africa (Gorman et al., 1983). More recently, BTV-22 and BTV-24 have also been isolated from South Africa (Roy, 1989).

In the U.S., BTV-10 was first isolated in sheep in 1952 (Howell, 1960). BTV-11 and BTV-17 were also isolated in sheep in 1955 and 1962, respectively (Barber, 1979). In 1967, BTV-13 was isolated from bovine blood and found to be serologically distinct from the other three. In 1982, BTV-2 was isolated from sentinel cattle near Ona, Florida (Gibbs et al. 1983). Two electropherotypes of BTV-2, Ona A and Ona B were identified from these cattle (Collisson et al., 1985). Ona A is indistinguishable from BTV-2 isolated in Africa in 1959 and is postulated to have been introduced to Florida by infected *Culicoides* from Cuba carried on the wind (Sellers, 1989). Ona B may be a variant of Ona A, evolving as a result of mutation or reassortment with another BTV strain (Collisson et al., 1985).

2. BTV morphology and stability

Three morphologically different BTV particles have been identified: virions, core particles and subcore particles

(Huismans and van Dijk, 1990). Like other orbiviruses, BTV has large, doughnut-shaped capsomeres on the nucleocapsid surface (Latin *orbis* = ring or circle) (Knudson and Monath, 1990). Treatment of the virion with chymotrypsin removes the outer capsid polypeptides resulting in the formation of a core particle with associated transcriptase activity (Van Dijk and Huismans, 1980). When VP7 is removed, core particles can be converted to subcore particles that are very unstable (Huismans and van Dijk, 1990). BTV can either exist as unenveloped core particles or as more complex membrane-bounded forms (Bowne and Ritchie, 1970). The core particles have a layer of fine surface projections that may be covered by a membrane derived from the host cell. Several core particles can be enclosed in a single membranous sac.

The virus is stable under average environmental temperatures and relatively resistant to ether, chloroform and sodium deoxycholate (Borden et al., 1971). BTV can be held in OCG (potassium oxalate, phenol, and glycerin) at room temperature for long periods of time. Like reoviruses, BTV is resistant to RNase degradation, but BTV is smaller (54um versus 76um) and has fewer capsomeres (32 versus 92) (Els and Verwoerd, 1969). Freezing slowly at -10°C to -20°C is deleterious to the virus (Luedke et al., 1985).

3. Genomic organization and coding assignments

The BTV genome consists of 10 double-stranded RNA (dsRNA) segments that code for 10 virus-specified polypeptides (Gorman et al., 1983). Gene coding assignments have been determined by *in vitro* translation of each dsRNA segment after denaturation (one strand is a functional mRNA) (Knudson and Monath, 1990). Most of the segments of BTV serotypes found in the U.S. have been sequenced (Table 1.1). Some segments from Australian serotypes BTV-1 and South African serotype BTV-1 also have been sequenced (Gould, 1988a,b; Yamaguchi et al., 1988b; Gould et al., 1988; Gould, 1987; Gould and Pritchard, 1988; Gould, 1990; Wade-Evans, 1988; Wade-Evans, 1990a,b; Wade-Evans, 1992). Each of the 10 RNA segments has highly conserved terminal sequences (5' end of the mRNA sense strand: GUUAAA; 3' end of the mRNA sense strand: CACUUAC) (Fukusho et al., 1989). These terminal sequences serve as recognition sites for initiation of transcription by the viral transcriptase, signals for RNA replication, and genome assembly (Rao et al., 1983).

Genomic numbering assignments are based upon the order of migration of the dsRNA segment in an agarose gel electrophoresis system (Pedley et al., 1988). However, the dsRNA segments may migrate differently in a polyacrylamide gel electrophoresis system (Mertens et al., 1984).

Table 1.1: Published sequences of BTV RNA segments of U.S. serotypes

Segment	BT-2	BT-10	BT-11	BT-13	BT-17
1					
2	Yamaguchi et al. 1988a	Purdy et al. 1985	Ghiasi et al. 1987	Fukusho et al. 1987	Ghiasi et al. 1987
3	Hwang et al. 1994	Ghiasi et al. 1985			Purdy et al. 1984
4		Yu et al. 1988			
5	Hirasawa and Roy 1990	Purdy et al. 1986	Dunn et al. 1991	Oldfield et al. 1991	
6	Hwang et al. 1992	Lee and Roy 1987	Yang and Li 1992	Wang et al. 1989	
7	Kowalik & Li 1991	Yu et al. 1988	Kowalik et al. 1990	Kowalik and Li 1989	Grubman and Samal 1989
8	Yang et al. 1992	Fukusho et al. 1989		Grubman et al. 1990	
9	Hwang et al. 1992a	Fukusho et al. 1989	Hwang et al. 1992a	Hwang et al. 1992a	Hwang et al. 1992a
10	Hwang et al. 1992b	Lee and Roy 1986	Hwang et al. 1992b	Hwang et al. 1992b	Hwang et al. 1992b

Cells infected with BTV contain three major non-structural proteins (NS1, NS2, and NS3/NS3A) and seven structural proteins (VP1, VP2, VP3, VP4, VP5, VP6, and VP7). The coding assignments of BTV proteins and corresponding molecular weights, locations of the proteins, and coding segment sizes are shown in Table 1.2 (Huismans and Van Dijk, 1990).

Table 1.2. RNA Coding Assignments of BTV Proteins

PROTEIN	MOLECULAR WEIGHT(kd)	LOCATION	CODING SEGMENT	SEGMENT SIZE(BP)
VP1	149.5	CORE	1	3954
VP2	111.0	OUTER CAPSID	2	2926
VP3	103.3	CORE	3	2760
VP4	76.4	CORE	4	2011
VP5	59.1	OUTER CAPSID	5	1638
NS1	64.4	INFECTED CELL	6	1769
VP7	38.5	CORE	7	1156
NS2	40.9	INFECTED CELL	8	1124
VP6	35.7	CORE	9	1046
NS3/NS3A	25.6	INFECTED CELL	10	822

4. BTV proteins

Two of the major polypeptides, VP2 and VP5, form the diffuse layer surrounding the nucleocapsid. The nucleocapsid contains five polypeptides, VP1, VP3, VP4, VP6, and VP7 (Verwoerd et al., 1972). VP1 protein has been expressed in *Spodoptera frugiperda* cells by a recombinant baculovirus and is associated with RNA-directed RNA

polymerase activity (Urakawa et al., 1989). BTV serotype-specific and neutralizing epitopes are associated with VP2 (Kahlon et al., 1983). Proposed roles for VP2 include serotype specificity, neutralizing antibody induction, hemagglutination (Cowley and Gorman, 1987; Hwang and Li, 1993). VP2 may be the major target for cytotoxic T-cells (Schultz and Grieder, 1987). VP3, a major structural core protein contains group-specific antigenic determinants (Huismans and Erasmus, 1981). VP3, present in far fewer copies than VP7, plays a crucial role in the assembly of virions of the correct diameter by forming a loose scaffold onto which VP7 is attached (Basak et al., 1992). VP4 has GTP-binding activity (LeBlois et al., 1992) and contains a leucine zipper motif near its carboxyl terminus (Huang et al., 1993). VP5, like VP2, is also a serotype-specific protein, but is more highly conserved between serotypes than VP2 (Wade-Evans et al., 1988). VP5 plays a minor role in serotype specificity along with VP2 (Mertens et al., 1989) and may play a role in eliciting protective immunity. VP5 was recently found to be glycosylated and should be termed GP5 due to the presence of a carbohydrate chain (Yang and Li, 1993). NS1 can also be expressed by recombinant baculoviruses (Urakawa and Roy, 1988) and is associated with numerous tubular structures. NS2 is a phosphorylated protein (Huismans et al., 1987b) and may be used to elicit

cell-mediated immunity, which could provide a means for cross serotypic protection (Devaney et al., 1988). NS2 also has the ability to bind single-stranded RNA (Wade-Evans, 1992; Hall et al., 1989). In laboratory studies, NS1 is translated *in vivo* in the largest amount, but *in vitro*, NS2 is the most efficiently translated protein (Van Dijk and Huisman, 1988). VP6, a minor component of the core, is believed to be a nucleic-acid binding protein associated with the viral RNA in the cores of virions (Roy et al., 1990a).

VP7 is a group-specific antigen that is used serologically to diagnose BTV infection. BTV antibodies specific for VP7 are predominantly responsible for the precipitin line that is formed in a standard AGID test (Adkison et al., 1987). Two minor nonstructural proteins, NS3 and NS3A, previously designated VP8 and VP8A (Sanger and Mertens, 1983), are glycosylated (Wu et al., 1992). Virus-like particles of BTV have been shown to be released in the presence of NS3/NS3A, but not when the NS1 protein is coexpressed (Hyatt et al., 1993). NS3/NS3A is required for the budding and release of VLP's consisting of VP2, VP3, VP5, and VP7 from infected cells (Hyatt et al., 1993).

5. Replication

BTV replicates in a wide variety of vertebrate and invertebrate cells *in vivo* and *in vitro*. Absorbed virus

particles can be observed on cell surfaces within minutes after initial infection (Gorman et al., 1983). BTV depends upon a receptor-mediated endocytotic process to infect cells. Within one hour after infection, the virion loses its major capsid proteins VP2 and VP5 and is converted into a core particle (Huismans et al., 1987c). Viral replication occurs in the cytoplasm and is associated with the development of viral matrices, macrotubules and aggregates of mature virions (Howerth and Tyler, 1988). The ten double-stranded RNA segments are transcribed into ten single-strand mRNA's (Huismans and Verwoerd, 1973). Six hours after infection, core particles are converted to skeleton-like subcore particles that contain only VP1, VP3, VP4, VP6, and all 10 double-stranded RNA segments (Huismans et al., 1987c). BTV can then be released as either enveloped particles, by budding through the plasma membrane, or as nonenveloped particles, by extrusion through the membrane (Hyatt et al., 1989).

6. Evolution

RNA genomes, because of their high mutational frequencies, exhibit considerable genetic diversity and rapid evolution. Genetic diversity in BTV is thought to be the accumulation of point mutations in the genome (genetic drift) (Sugiyama et al., 1982) and/or reassortment events between segments of the genome (Barber and Collisson, 1985).

The genetic heterogeneity of BTV has been demonstrated using a variety of procedures such as oligonucleotide fingerprint analysis (Sugiyama et al., 1981; Rao and Roy, 1983; Collisson et al., 1989) and nucleic acid hybridization techniques (Ritter and Roy, 1988). Due to the segmented nature of the BTV genome, polyacrylamide gel electrophoresis (SDS-PAGE) gels of the viral dsRNA can be used to observe migration profiles of the 10 segments (Knudson et al., 1982). The electropherotype of a BTV strain is characteristic for that strain, but not necessarily for all the strains of a specific serotype. These profiles can be used to identify BTV diversity and genome reassortment (Knudson et al., 1982; Dunn and Stott, 1989).

In cells simultaneously infected with two or more related viruses, independent reassortment of parental genes results in recombinants. This phenomenon has been demonstrated between serotypes of the BTV, Eubanangue, and Wallal serogroups of orbiviruses (Gorman et al., 1983). High frequency reassortment of BTV serotypes can occur in dually infected vectors (El Hussein et al., 1989b) and bovine vertebrate hosts (Stott et al., 1987), while low frequency reassortment is seen in ovine vertebrate hosts (Samal et al., 1987a). Reassortment has been found to be an early event in the BTV replication cycle suggesting that only a single round of reassortment occurs per progeny

(Ramig et al., 1989). Mixed infection of 11 *C. variipennis* with BTV-10 and BTV-17, resulted in high frequency reassortment of viral genome segments (8 flies produced virus progeny with reassortant electropherotypes) (Samal et al., 1987b).

In vivo, mixed infections do occur in the field as shown by the isolation of two or more BTV serotypes from the same animal, which could result in reassortment (Appleton and Letchworth, 1983). Multiple serotypes of BTV (serotypes 10 and 17; serotypes 11 and 17) have been recovered from infected sheep and cattle that suggest a mechanism for evolution of the various serotypes (Stott et al., 1982; Oberst et al., 1985). Multiple orbivirus serotypes including one serotype of BTV and two serotypes of EHDV have been recovered from a single heifer (Foster et al., 1980b). Segment reassortments can occur also in the bovine during mixed infection with strains of the same BTV serotype such as two strains of BTV-11 (Oberst et al., 1987). BTV-17 is believed to have evolved in the U.S., because it has not been found elsewhere. BTV-17 is most closely related to BTV-10 and BTV-11 and may have evolved through a combination of genetic drift and genomic reassortment (Kowalik and Li, 1991; Rao and Roy, 1983). BTV-11 strain UC8 originated through a reassortant event between BTV-10 and BTV-11 (de Mattos et al., 1991).

D. Bluetongue Virus Pathogenesis In Vertebrate Hosts

The pathogenesis of BTV in the vertebrate host is very different from that in the vector. The route of infection, viral spread in the body, and virus shedding are distinctly different. This difference in pathogenesis could be due in part to different neutralizing epitopes of BTV that are host-dependent (Greider and Schultz, 1991). Both the major (VP2) and the minor (VP5) outer coat proteins contribute neutralizing determinants, however, VP5 may have a unique function in the infectivity in insect cells while VP2 may function in the infectivity in vertebrate cells (Greider and Schultz, 1991). A similar situation is seen in LaCrosse virus, where the small envelope glycoprotein is important for insect target cell attachment, while the large envelope glycoprotein is the attachment protein to mammalian vertebrate cells (Ludwig et al., 1989).

1. General infection and replication in vertebrate hosts

After being injected into a vertebrate host by the bite of an infected vector, BTV enters the regional lymph nodes and is disseminated via lymph and/or the bloodstream throughout the body to the lymphoid tissues where further replication occurs. BTV is then carried in the bloodstream to vascular endothelial cells and subsequently infects the majority of the body tissues (Pini, 1976). BTV infection

and multiplication occur in the cytoplasm of vascular endothelial cells. After virus particles are released, a cell-associated, usually erythrocytic, viremia occurs, resulting in fever, with peak viremic titer coinciding with the highest temperature (Whetter et al., 1989). The sequestration of the virus within red blood cells (RBC) could contribute to the prolonged viremia reported in some infected vertebrate animals (Nunamaker et al., 1992). The prolonged viremia is thought to be due to the adsorption and subsequent infection (but not replication) of the circulating red blood cells that have a long half-life and not the continued replication of the virus in the body tissues (MacLachlan et al., 1990). This cellular association impedes prompt elimination of virus by the immune system.

a. Ovines

The virus is more pathogenic in sheep than in cattle, and infection can result in 50% mortality rates and 80-100% morbidity rates (Holbrook, 1988). Sheep vary in their genetic susceptibility to BTV infection. In Africa, mortality rates can be as high as 90%, whereas, in the U.S., mortality rates are usually less than 10% (Moulton, 1961). In suckling lambs, morbidity and mortality rates may be as high as 80 and 100 percent, respectively (Eisa et al., 1980). Incubation periods average 6-7 days. The first

clinical sign is usually a rise in body temperature up to 105°F, followed by anorexia and stiffness. The tongue may become cyanotic in severe cases due to facial and buccal edema, therefore the name "bluetongue". "Wool break" due to hyperemic skin can develop resulting in the shedding of the fleece (Uren and Squire, 1982). Animals may experience difficulty in swallowing due to mouth lesions that vary from small ulcerations to complete sloughing of the mucosa of the lips and muzzle (Gibbs and Greiner, 1988). Mucopurulent nasal discharge, lacrimation and frothing saliva encrust the muzzle. Edema in the hooves leads to coronitis and marked lameness. Different breeds of sheep vary in their susceptibility to BTV and older sheep give a more severe clinical response than lambs (Hourrigan and Klingsporn, 1975b).

Under natural conditions, Spruell (1905) did not believe that goats were susceptible to BTV infection, but BTV can persist for 20 days in inoculated goats. Natural infection of goats has been documented in Israel and in Spain, and in the latter only mild disease with no mortality has resulted (Lopez and Botija, 1958). However, in India both clinical BT disease and mortality in goats have been observed (Sapre, 1964). In one region in India, almost 18% of the goats were found to be BTV seropositive, indicating that goats may be an important link in the epidemiology of

the disease (Dubey et al., 1988). Through the 1970's, clinical disease was rarely reported in goats in the U.S. (Luedke and Anakwenze, 1972); in one animal a high BTV titer leading to a progressive anemia was documented. In 1980, twenty-six isolates of BTV-10, -11, and -17 were obtained from goats in California (Inverso et al., 1980). These goats showed clinical signs ranging from abortion to pneumonia to swollen joints. In Louisiana, 18.4% of the goat herds (7/38) and 2.8% of the individual goats were BTV-seropositive (Fulton et al., 1981). BT-4 has recently been isolated from goats in the Sultanate of Oman (Al-Basaidy and Mellor, 1991).

b. Bovines

BTV infection in cattle is usually asymptomatic and fewer than 10% show actual clinical signs (Luedke and Jones 1984). BT disease in cattle can be confused with infectious bovine rhinotracheitis, foot and mouth disease, vesicular stomatitis, and bovine viral diarrhea (Bowne, 1973). An underlying effect of BTV infection can be the loss of milk production and an increased susceptibility to secondary infections. Excessive salivation, fever, edema of the mouth, laminitis and coronitis can be associated with bovine infection (Gibbs and Greiner 1988). Dermatitis and stomatitis symptoms may be mediated by IgE (type I hypersensitivity) (Anderson et al., 1987). Infection of the

fetus can result in abortion and teratogenesis (Gibbs and Greiner, 1988).

BTV viremia is frequently prolonged in infected cattle, sometimes as long as four months (Hourrigan et al., 1975a). BTV and specific neutralizing antibody may circulate together for several weeks in the peripheral blood (MacLachlan et al., 1987; Richards et al., 1988). This ability to persist in cattle despite a strong humoral immune response is not due to antigenic drift, as the BTV genome remains stable throughout the infection (Heidner et al., 1988). Instead, it has been postulated that BTV may reside in a circulating cell type and remain protected from the effects of neutralizing antibody (MacLachlan et al., 1987). A similar mechanism has been identified for Colorado tick fever virus that infects erythrocyte precursors in the bone marrow and produces a prolonged infection despite presence of neutralizing antibody in the serum (Emmons et al., 1972).

c. Wild ruminants

Geographic distribution of BT disease in wild ruminants generally parallels that of domestic livestock (Drolet et al., 1990). Twenty-four percent (128/528) of the deer in western Nebraska were serologically positive for BTV antibodies (Johnson et al., 1986). In white-tailed deer and pronghorn antelope, infection may be rapidly fatal due to disseminated intravascular coagulation associated with

degenerative changes and necrosis of the blood vessel walls (Hoff and Hoff 1976). At least 3,200 pronghorn antelope were reported to have died of BTV infection during early fall of 1976 in eastern Wyoming (Thorne et al., 1988). Another BTV epizootic in late summer of 1984 in northeastern Wyoming resulted in 300 known pronghorn deaths (Thorne et al., 1988). BTV-17 was isolated from pronghorns in both cases. Signs of BT disease in antelope include anorexia, central nervous system depression, incoordination, dyspnea, and pneumonia (Hoff and Trainer, 1972). Serological studies have revealed evidence of BTV infection in elk, bighorn sheep, Barbary sheep, moose, and several species of deer (Trainer and Jochim, 1969; Clark et al., 1985; Couvillion et al., 1980). BTV isolations have also been made from the Reeve's muntjac and the greater kudu (Hoff et al, 1973). In the laboratory, BTV infection of white-tailed deer can also result in a subclinical form of the disease accompanied by a persistent viremia lasting at least 13 days (Thomas and Trainer, 1970a,b). Experimentally-infected deer can remain seropositive for a year or more (Work et al., 1992). In 1989, 100% of the over 24 month-old bison surveyed in Kansas were BTV seropositive, a great increase from 38% in 1987. Antibodies to BTV were found recently in captive oryx from the U.S. and Saudi Arabia (Greth et al., 1992).

d. Other vertebrate hosts

A serological survey for BTV antibodies in Nigeria revealed that 17% of the camels in the study (11/64) were seropositive (Exeifeke et al., 1983). BTV has been isolated from a vleisrat (*Otomys irroratus*) and two striped mice (*Rhabdomys pumilio*) (Neitz, 1966). Dogs, cats, ferrets, rabbits and guinea pigs were not susceptible to BTV infection (Howell, 1963)

2. **Transplacental transmission**

BTV can be transmitted by a viremic dam to her unborn offspring. The occurrence of abortion, teratology or other sequelae appears to be time-dependent.

a. Ovines

When ewes were inoculated with BT virus at their 5th or 6th week of gestation, 30% transplacentally transmitted the virus to their offspring (Anderson and Jensen, 1969). Embryos from BTV-infected donor ewes bred by BTV-infected rams can be transferred to BTV-seronegative recipient ewes. Neither the recipient ewes nor the transferred embryos show BTV seroconversion (Hare et al., 1988). The investigators in this study concluded that the risk of transmitting BTV by embryo transfer was minimal, provided that the embryos are properly washed between collection and transfer.

Cerebral malformations of fetal or newborn lambs have been described in the field both after natural infection

(Schmidt and Panciera, 1973) and after experimental use of BTV vaccine (Osburn et al., 1971). These neurologic malformations include porencephaly and cerebral necrosis, and are age-dependent. Fetuses infected at 50 days of gestation are afflicted with necrosis of undifferentiated cells of the brain while those infected over 100 days of gestation show only chronic inflammation (Waldvogel et al., 1987). Congenital abnormalities of the fetus due to BTV-infection of the ewe during pregnancy include skeletal deformities such as curved limbs, kyphosis or curved backs, blindness, and microencephaly (Sharma et al., 1986).

b. Bovines

Transplacental transmission of BTV in bovines remains a controversial area of discussion. In cattle, the intact *zona pellucida* of the embryo prevents infection. Interestingly, laboratory studies demonstrate that large numbers of BTV virions adhere to the surface of the *zona pellucida* of exposed embryos (Gillespie et al., 1990). Under standard conditions, transfer of embryos from BTV-infected donor cattle to BTV-seronegative recipients does not result in transmission of BTV (Bowen et al., 1983b). The placental membrane does not prevent BTV infection of the developing embryo (13-45 days) or fetus (over 46 days) and (Hubbert et al., 1972). Therefore, the fetus may be killed and reabsorbed or expelled, or it may recover with

subsequent teratogenetic malformations. The fetus also may become a persistent carrier of the virus throughout its life and may remain immunologically unresponsive (Luedke, 1985). BTV infection of fetuses in late gestation can result in premature birth resulting in small, weak calves (Waldvogel et al., 1992). Hydrencephaly has been documented in the field (McKercher et al., 1970) and been produced experimentally by intra-uterine infection (Barnard and Pienaar, 1976). Direct inoculation of BTV into 125-day old fetuses resulted in the development of hydrencephaly at birth (MacLachlan and Osburn, 1983).

3. Venereal transmission

Infected bulls can transmit BTV to uninfected cows or progeny by venereal transmission via contaminated semen. BTV has been isolated from semen samples using both cell culture and embryonated chicken eggs (ECE) (Breckon et al., 1980). This has caused great international concern in the cattle industry and may be the biggest barrier to exportation of cattle and semen from the US. Abnormalities and BTV-like particles have been observed in spermatozoa from BTV-infected bulls (Foster et al., 1980a). In no instance, however, has virus been isolated from semen without concurrent isolation from blood (Bowen et al., 1983a). The latter investigators concluded that attempting to isolate BTV from the blood, rather than from semen, would

be a more reliable indicator of possible BTV contamination in the semen. Attempts to isolate BTV from 85 semen samples from 12 seropositive bulls on 2 types of cell cultures were all negative (Phillips et al., 1986), indicating that persistent infection of BTV in the semen was unlikely. However, some studies suggest that virus isolation by cell culture techniques is not as sensitive as inoculation of live sheep to identify BTV in semen (Parsonson et al., 1981). Nonetheless, in a five year period, no evidence of BTV contamination of semen was detected in 18 naturally-occurring BTV infections in bulls (Gard et al., 1989).

E. Bluetongue Virus--Vector Associations

BTV is maintained in nature in a cycle involving biting midge arthropod vectors and ruminant vertebrate hosts. The arthropod vector is a significant part of the virus cycle, because it is the primary means of virus dispersal and a mechanism for virus amplification. The vertebrate host acts as a site of viral replication and amplification. Multiple vectors may become infected while feeding on a viremic host.

1. Arbovirus cycle

A simple arbovirus cycle consists of a hematophagous arthropod and a susceptible host vertebrate, which develops a viremia but not necessarily clinical disease. A susceptible vector, after feeding on the host, becomes infected with the virus and after replication, transmits the

virus to another susceptible vertebrate host. More complicated arbovirus cycles involve dead-end hosts that may exhibit clinical disease but do not contribute to virus amplification by infecting susceptible insect vectors. Other cycles, such as that for yellow fever virus, have different insect vectors and host animals depending on the geographical region of transmission.

2. *Culicoides variipennis*

a. *C. variipennis* biology

More than 1000 species of *Culicoides* have been identified world-wide, but only a few have been linked with the transmission of BTV (Mellor, 1992). *Culicoides variipennis* (Diptera: Ceratopogonidae) is the primary vector of BTV in the U.S. The range of *C. variipennis* in western North America extends into both Canada and Mexico but rarely into the far northeastern and southeastern U.S. (Jones et al., 1981). *C. variipennis* was incriminated as the vector based on virus isolations from field-caught flies (Price and Hardy, 1954) and by laboratory biological transmission studies. Five sub-species have been described in the U.S.: *C. v. variipennis* (Northeast), *C. v. sonorensis* (Southwest), *C. v. occidentalis* (Far West), *C. v. australis* (Southcentral), and *C. v. albertensis* (Northcentral) (Tabachnick, 1992).

Culicoides variipennis breed in muddy areas in pastures contaminated by waste effluent or decaying vegetative material. In Colorado, *C. variipennis* emerge from mid-March to October, and overwinter as larvae. Most epidemics of BTV in the U.S. occur in late summer in the U.S., at the end of the *Culicoides* breeding season. The life cycle of *Culicoides* includes four stages: egg, larval, pupal, and adult. Eggs are laid in batches ranging from 30-450 and hatch within 2-4 days. After hatching, the *Culicoides* larvae pass through four instars. *C. variipennis* overwinter as 4th instar larvae in Colorado and survive by burrowing down deep into the mud of the breeding site. Larvae are most abundant in the surface layers of mud extending 7-10 cm above the shoreline to 30cm below and can be found as deep as 5 cm in the mud. The larvae swim with an oscillatory motion enabling them to move throughout the mud easily, and are rarely found swimming freely in the water. The fourth instar gives rise to the pupal stage which is a short-lived non-feeding stage. This gives rise to the winged adults, which average 0.77 mm in length (Blanton and Wirth, 1979).

During oogenesis, *C. variipennis* produce and accumulate externally visible pigmentation granules in their abdomen that can be used to categorize them as nulliparous or parous (Akey, 1987). Females are designated as gravid if they have either well-developed oocytes or eggs externally visible in

the abdomen, which usually occurs at least 3 days after a blood meal (Akey and Barnard, 1983). Most species of *Culicoides* have dark and light patterned wings. This pattern is due to pigmentation in the wing membrane, which can fade in specimens stored in alcohol or exposed to light. Both rearing temperature and larval density have an effect on longevity, size and fecundity of *C. variipennis* (Akey et al., 1978).

b. *C. variipennis*-virus interaction

C. variipennis is an efficient vector of BTV. Once infected, the midges remain infected for life (several weeks). BTV multiplies in the vector up to a titer of approximately 4 to 5 $\log_{10}$ TCID₅₀/fly over a period of eight days (Jennings and Boorman, 1980). Refractory midges are not competent vectors to transmit the virus because of various barriers to replication, and in incompetent vectors BTV becomes undetectable by 2-3 days post-ingestion (Mellor, 1990). Most *Culicoides* species disperse only short distances from their breeding sites but, flight range studies of *C. variipennis* females found average flights per night of 1.89 km with a maximum flight of 4.0 km (Lillie et al., 1981). The midges can fly, can be windblown, or can be transported by vehicle or aircraft for great distances, disseminating BTV to new geographical areas. *Culicoides* species feed on a wide range of hosts including humans,

ruminants, birds, turtles, lizards, and even engorged mosquitoes (Hair and Turner, 1968), but usually prefer only one host. Once an infected bloodmeal is ingested by the *Culicoides*, BTV attaches to the luminal surface of the mid-gut cells and infects them (Mellor, 1990). After replication in the mid-gut cells, the virus is released into the hemocoel with replication occurring in the salivary glands of the *Culicoides* (Holbrook, 1988). The time from virus infection of the vector until virus can be transmitted (the extrinsic incubation period) lasts 10-15 days at 25°C. In one study a direct immunofluorescence test detected virus antigen in the midgut after six days of incubation and by 10 days antigen could be found in the head (Chandler et al., 1985). Other temporal limits on BTV dissemination and infection of secondary target organs have been described (Ballinger et al., 1987). During subsequent bloodmeals, the virus is transmitted to new vertebrate hosts.

Many studies of BTV transmission by *C. variipennis* have been done with laboratory colonies. Work with these colonies, however, may not adequately describe the vector competence of natural populations (Jones and Foster, 1978). Oral susceptibility to infection of *C. variipennis* with BTV may be under the control of a single genetic locus (*blu*) (Tabachnick, 1991a). In the laboratory, *C. variipennis* has been shown to be a capable vector of Akabane virus

(Jennings and Mellor, 1989), African horse sickness virus (Boorman et al., 1975, Brown and Dardiri, 1990), *Onchocerca cervicalis* (Collins and Jones, 1978), main drain virus (Mellor et al, 1974) and epizootic hemorrhagic disease virus (EHDV) (Wilson, 1991).

b. *C. variipennis* collection

To study field populations of *Culicoides*, various traps can be employed either to capture the vectors alive for later virus isolation or to preserve the vectors in some sort of fixative such as polyethylene glycol. Traps such as the New Jersey trap and the CDC trap include an incandescent light source, a suction fan and a net or jar to collect the insects in (Holbrook and Bobian, 1989). The catches of *Culicoides* are directly proportional to the strength of the light source. A strong light source attracts a higher percentage of gravid flies (those having blood fed at least once) than non-gravid flies (Holbrook et al., 1985). New Jersey light traps with dry ice as a carbon dioxide bait collect significantly more nulliparous flies and parous *C. variipennis* (those searching for a blood meal) than traps without the dry ice (Wieser-Schimpf et al., 1991). New Jersey light traps without the dry ice collect the highest proportion of flies that have taken at least one bloodmeal; this is the most useful when trapping to determine BTV prevalence rates in the vector (Holbrook and Bobian, 1989).

3. *C. insignis* and other *Culicoides* vectors

Culicoides insignis has been implicated as the arthropod vector in the transmission of BTV-2 in Southern Florida and the Caribbean (Gibbs et al., 1983). *C. insignis* has been shown to be a biological vector of BTV in the laboratory by being capable of transmitting BTV to susceptible sheep (Tanya et al., 1992). *Culicoides boydii* has recently been suspected as a potential vector of BTV to desert bighorn sheep in California (Wirth and Mullens, 1992). In the Middle East and most of Africa, the major vector of BTV is *Culicoides imicola* (Al-Basaidy and Mellor, 1991; Boorman, 1986). *C. imicola* is also the main vector of BTV in Spain and Portugal (Mellor et al., 1985).

Culicoides pallidipennis is a major vector of BTV in South Africa (Nevill, 1971). In South Africa, cattle are maintained in close proximity to sheep because of the apparent host preference of *C. pallidipennis* for cattle, thereby reducing the incidence of BTV infection of sheep (Erasmus, 1975b). BTV has been isolated from *C. obsoletus* in Cyprus (Mellor and Pitzolis, 1979). In the laboratory, both *C. debilipalpis* and *C. stellifer* become infected with BTV after intrathoracic inoculation and membrane feeding of BTV, respectively. These two vectors may play a role in the transmission of BTV between cattle and wild deer (Mullen et al., 1985). *C. nubeculosus* and *C. impunctatus* were found to

support BTV multiplication after ingestion and may act as potential vectors of BTV in geographical areas that are presently BTV-free (Jennings and Mellor, 1988).

4. Alternate vectors of BTV

BTV has been isolated from anopheline mosquitoes collected in Indonesia in 1980 and 1981 (Brown et al., 1992). BTV has also been isolated from naturally infected cattle lice (*Haematopinus eurysternus*) (Hoff and Hoff, 1976), and the common sheep ked, *Melophagus ovinus* has also been incriminated as a vector (Luedke et al, 1965). The argasid tick, *Ornithodoros coriaceus*, can become infected with BTV in the laboratory and has been reported to transmit the virus to a susceptible cow at 42 days post ingestion of the infected blood meal (Stott et al., 1985a). Infectious virus is present in the salivary glands of infected ticks, suggesting that this tick has the potential to serve as a biological vector of BTV. BTV has been isolated from ticks of the *Ixodidae* family in the Republic of Guinea (Konstantinov, 1990).

BTV may also be transmitted without a vector. Sheep can become infected by repeated oral administration of BTV; it has also been postulated that laboratory control sheep can be infected by oral ingestion of virus in urine-contaminated water or feed (Jochim et al., 1965). The significance of this in nature is unknown.

F. Bluetongue Virus Transeasonality

BTV is a seasonal disease in temperate climates; circulating only when environmental conditions are favorable for the vector hosts. However, in spite of this seasonality of the vector species, BTV remains endemic in the same areas year after year. The true mechanism of this transeasonality or overwintering of BTV remains to be elucidated. Proposed mechanisms to account for BTV transeasonality include: Persistence in the vertebrate host, transplacental transmission, continuous BTV cycle, and vertical transmission in vectors.

1. Persistence in the vertebrate host

Persistent infection of vertebrates has been proposed as an overwintering mechanism. One persistently infected bull, when stimulated by uninfected gnat bites, developed a viremia "shower" capable of infecting subsequently feeding gnats (Luedke et al., 1977a). Based upon this report, it has been proposed that latently infected cattle are the reservoir for BTV during the winter, and become viremic after stimulation by *Culicoides* bites. In sheep and goats, however, viremia in adults has not been observed past 50 days (Gibbs et al., 1979).

BTV infection of bovine kidney cells (MDBK) can be regulated by the incubation temperature to be either persistent (low levels of virus), lytic (high levels of

virus) or latent (no virus produced) (Hallum et al., 1984). The persistent and latent stages can be reversed, and latent infections are only achieved when the incubation temperature is 41°C. Since cattle have higher body temperatures (ranging up to 39.5°C) this observation may play a role in the postulated latent infection (Hallum et al., 1984).

2. Transplacental transmission

Transplacental infection as a method of overwintering has been investigated in cattle (Luedke et al., 1977b) and in sheep (Gibbs et al., 1979). Approximately 40% of the calves born to infected heifers become persistently infected with BTV, while 80% of the lamb fetuses are infected. However, if infected early in gestation, gross congenital abnormalities result both in calves (Thomas et al., 1986) and in lambs (Richardson et al., 1985)

After birth, virus can be isolated from the peripheral blood of persistently infected calves,, but neutralizing antibody or antibody to group-specific antigens remains undetectable. Thus, immune tolerance may permit cattle to serve as a mechanism for overwintering. However, this tolerant state has not been reproducible (Thomas et al., 1986), even after intrauterine virus administration (MacLachlan et al., 1984).

To cause a persistent infection of the lamb fetus, the ewes must be infected between 61 and 74 days gestation

(Gibbs et al., 1979). The viremia titer in the neonate is typically low and present for only a short time (Gibbs and Greiner 1988).

3. Continuous BTV cycle

Climactic conditions in South Africa may permit the summer cycle between *Culicoides* and cattle to be continued at a lower level in the winter (Nevill, 1971). Because of its presence throughout the year in Israel, *C. imicola* has been implicated as an overwintering vector of BTV (Braverman 1985). In southern California, *C. variipennis* are active over the whole 12 months of the year but are less abundant in late winter (Mullens, 1985). Gravid females are also collected in southern California year-round, which suggests some level of successful blood feeding. Such mechanisms would not be possible in temperate regions where low temperatures would preclude any winter vector activity. However, *Culicoides* adults can survive for 53 days when refrigerated at 4°C and survive outdoors during winter in South Africa for up to 51 days (Nevill, 1971), so this hypothesis should not be underestimated in some of the warmer temperate regions.

4. Vertical transmission in vectors

Interest in vertical and transovarial transmission (TOT) of arboviruses by the vector host has increased recently. TOT is defined as the transmission of a virus

from an infected female to her offspring via the ovary (Turell 1988). TOT has been demonstrated to be the overwintering mechanism of some arthropod-borne viruses such as LaCrosse virus in *Aedes triseriatus* mosquitoes (Watts et al., 1973). Transovarially-infected progeny emerge the next season and transmit virus by bite to susceptible vertebrate hosts (Turell 1988).

Jones (1985) proposed that BTV may be maintained by TOT in the vector. TOT is an attractive hypothesis for overwintering. However, initial experiments were unable to demonstrate TOT of BTV in egg batches of infected females (Jones and Foster, 1971). Flies from the parent colony (established in 1957) were fed on infected blood meals and then subsequent uninfected blood meals. None of their progeny yielded infectious BTV virus. Direct immunofluorescence techniques revealed that BTV antigen was detected in ovarian sheath tissues of *C. variipennis*, but not in the follicles or eggs (Ballinger et al., 1987). Flies were given only a single blood meal before dissection. Similarly, an immunogold labeling technique demonstrated that progeny flies of infected females were negative for BTV even after five gonadotrophic cycles (Nunamaker et al., 1990).

Nonetheless, these studies do not preclude vertical transmission as an overwintering mechanism for BTV for the

following reasons: All BTV studies were done with one strain of *C. variipennis* that has been colonized for a long period of time. Genetic "bottlenecking" could have yielded a strain incapable of TOT, or alternatively, the original colony material may have been incapable of TOT. Susceptibility to infection with BTV of *C. variipennis* from laboratory colonies should not be assumed to be constant, particularly when assessing vector efficiency (Jennings and Mellor, 1987). Genetic variability in such traits is well documented (Tesh, 1980). Studies by Jones and Foster (1978) demonstrated that colony populations may not represent the field populations from which they were derived. Differences between the field environment and the laboratory environment may play a role in TOT. Rearing temperature has been shown to have a drastic effect on TOT of St. Louis encephalitis virus in mosquitoes (Hardy et al., 1980). Minimum infection rates in adult progeny of infected *Aedes* mosquitos were significantly increased by rearing at 18°C rather than the usual 27°C. Further, it is now well recognized that vertical transmission of arboviruses is not restricted to TOT. Dengue viruses are transmitted via the micropyle to progeny mosquitoes during oviposition, presumably during insemination of the egg (Rosen et al, 1987).

Thus, although the previously cited BTV studies did not demonstrate viral antigen in the ovaries (Ballinger et al.,

1987; Nunamaker, et al., 1988), they do not preclude vertical transmission of BTV and overwintering in infected progeny. Finally, it is now recognized that unpassaged BTV isolates are extremely fastidious and unlikely to be detected in cell culture (i.e., Vero or BHK-21) isolation systems (Jochim, 1985). Thus, lack of evidence suggesting TOT in field studies may be due to false negative results.

G. Diagnosis of Bluetongue Virus Infections

Arboviral infections, including BTV can be diagnosed using a variety of techniques including virus isolation, viral antibody detection, host anti-viral antigen detection, and viral nucleic acid detection (Calisher, 1992). Conventional methods for diagnosis of arbovirus infections in vertebrates require demonstration of a serologic diagnostic change ($\geq$ 4-fold) in antibody titer between acute- and convalescent-phase serum specimens or isolation of virus by amplification in a bioassay such as susceptible cell cultures, animals, or mosquitoes and subsequent identification of the isolate by serologic techniques.

The conventional method for arbovirus surveillance in vectors involves isolation of virus from vectors or vector pools by amplification in a bioassay, followed by serologic identification. Test results typically are not available in time to permit practitioners to formulate a therapeutic plan

for the individual patient or institute the appropriate herd health control measures.

1. Virus isolation

BTV in diagnostic specimens can be isolated and amplified in a number of cell culture systems and laboratory animal hosts. In the past, BTV was propagated with some success in lamb kidney cells (Fernandes, 1959) and bovine bone marrow cells (Sawyer and Osburn, 1977). Today, various cell lines are used including primary fetal bovine and ovine kidney cells, a fetal tongue cell line derived from a bighorn sheep fetus (Castro et al., 1989), African green monkey kidney (VERO) cells, baby hamster kidney (BHK) cells, mouse fibroblasts (L-cells), and bovine pulmonary artery endothelial (CPAE) cells (Walton, 1980, Wechsler and McHolland, 1988).

Cell culture monolayers are grown to confluency in media such as minimal essential medium (MEM) supplemented with 5-10% fetal bovine serum (FBS). BTV produces cytopathic changes in infected cells including intracytoplasmic inclusion bodies (Browne and Jochim, 1967). BTV infectivity can be enhanced in cell culture by centrifugation (Sundin and Mecham, 1989). Supplemental calf serum should not be used until it is screened first for antibody specific for BTV, which is common in bovine serum

and can be transferred to the newborn via colostrum (Blanchard-Channell and Stott, 1989).

The mosquito, *Toxorhynchites brevipalpis* (Diptera: Culicidae), has been investigated in the laboratory as a propagation system for BTV like that used for dengue and other arboviruses (Jennings et al., 1983). BTV did multiply in the mosquito after intrathoracic inoculation, but there was no difference in sensitivity between this system and BHK cells. Non-mammalian cell lines from ticks, mosquitoes, and toads have been investigated for their use as virus isolation systems (Homan and Yunker, 1988). *Aedes albopictus* cells support the growth and multiplication of BTV with no obvious cytopathic effects. Cell lines developed from *C. variipennis* embryos (CuVa), support replication of BTV to high titer without discernible cytopathic effects (Wechsler et al., 1989). A dsRNA virus has since been detected in these cells (Wechsler et al., 1991) and designated CUV. No pathological effects of the virus have been observed in the CuVa cells.

Unpassaged BTV strains are difficult to propagate in conventional laboratory systems. Animal inoculation is the most sensitive procedure. BTV can be isolated by direct inoculation of the sample blood directly into susceptible sheep. Isolation of BTV in embryonated chicken eggs (ECE) is the gold standard. In the developing chicken embryo,

virus localizes in epithelial cells of the kidneys and lungs with smaller amounts of virus distributed in the rest of the organs and tissues (Wang et al., 1988). Intravenous inoculation of ten to thirteen day old embryonated chicken eggs are often used, especially for field isolates (Goldsmith et al., 1975; Goldsmith and Barzilas, 1968). This method has been found to be equal to sheep inoculation for the isolation of BTV (Foster et al., 1972). However, these conventional methods are labor-intensive, slow, and may require expensive and elaborate facilities.

2. Antibody detection

The detection of group-specific antibodies to BTV is an important method of screening animals for import or export and for monitoring seroprevalence in the field. Group-specific antibodies are detectable within 1-2 weeks of infection regardless of the presence or absence of detectable viremia (Roberts et al., 1992). Many more BTV diagnoses have been made by serologic detection than by isolation and identification of the virus (Jochim, 1985). In epizootic areas diagnosis can be made based on symptoms and the epizootic course. In BTV-free areas diagnosis must be confirmed by the detection of the virus or its antibodies in organ samples or serum. Many serologic tests have been adapted to detect antibodies to BTV. Complement fixation (CF) with hyperimmune serum and neutralization tests have

been used to identify BTV in isolates. The modified direct complement fixation (MDCF) in which the complement is supplemented by 5% non heat-inactivated, normal, pre-tested bovine serum is group-reactive and has been used in the past with some success (Boulanger and Frank, 1975). The agar-gel immunodiffusion (AGID) test is the most widely used assay for the detection of group-specific anti-BTV antibodies (Abu Elzein, 1984). Bluetongue immunodiffusion (BTID) is the official USDA-APHIS assay for testing sera for importation and exportation of domestic animals (Pearson and Jochim, 1979). However, it is not very sensitive or specific and the results are usually subjective. False negative results can occur due to the assay's limited sensitivity (Squire, 1989). False positive results are probably due to cross-reactivity with the antigenically similar EHD virus (Afshar et al., 1987a). Sequential infections with two or more of the BTV serotypes can lead to a broadly cross-reactive immune response (Elfatih et al., 1987).

Hemagglutination (Tokuhisa et al., 1981) and hemagglutination inhibition tests have been developed for BTV diagnosis (Van der Walt, 1980). However, the titers measured were generally 2-4 times lower than those measured by plaque neutralization; thus, the test was less sensitive for detecting low levels of antibody. Virus serum neutralization tests cannot only determine the presence of

BTV antibodies but can be also used to type BTV strains (Van der Walt, 1979). Plaque reduction neutralization and plaque inhibition tests have been used for serotyping isolates using cells such as BHK-21 (Jochim and Jones, 1976) and L cells (Davies and Blackburn, 1971). In a passive immunity cross-protection test, maternally-immunized mice have been used to serotype isolates and this method agreed with concurrent plaque reduction neutralization tests (Adu and Yuill, 1983).

Enzyme immunoassays (EIA) are based on specific antigen-antibody interactions and are characteristically very sensitive and specific (Charan and Gautam, 1984). Sensitivity is of fundamental importance to capture antigen EIAs because if sensitivity is adequate, there is no need to amplify the virus in a bioassay. Specificity of immunologic reagents is also important because it permits simultaneous detection and identification of the agent or antibody. Thus, EIAs preclude many difficulties associated with conventional serologic or virus isolation and identification techniques. Significantly, EIAs can produce diagnostic results rapidly and in clinical situations. Although EIAs are a major advance over conventional diagnostic procedures, some problems are inherent in the systems, including endogenous enzymes in the

sample, variable binding of immunoglobulins to the solid phase, and nonspecific protein binding.

As an alternative to AGID, enzyme-linked immunosorbent assays (ELISA's) have been developed using purified BTV antigen to quantitate antibodies in sera (Lunt et al., 1988a; Hubschle et al., 1981). Indirect ELISA's have been described (Manning and Chen, 1980), but these rely on a complicated purification procedure to produce the antigen, which may yield false positive results. Dot immunobinding assays (DIA) are easy to perform and if antigen-coated preblocked dipsticks are used, the whole test can be run in less than two hours (Gupta et al., 1990). Direct comparison showed DIA to be more sensitive than the indirect ELISA. Competitive ELISA's using a group-specific monoclonal antibody against BTV were found to be superior to both the AGID and the indirect ELISA tests (Afshar et al., 1989). The competitive ELISA has been recently designated as an official test for international monitoring of ruminants for BTV exposure (Afshar et al., 1992; Jeggo et al., 1992). Blocking ELISAs have been frequently used (Lunt et al., 1988b; Afshar et al., 1987b; Martyn et al., 1991) to increase the sensitivity and specificity of the ELISA assay by using monoclonal antibodies. By adding serial dilutions of bovine test sera containing BTV antibodies, the binding of a group-specific monoclonal to the BTV antigen is

inhibited. Antibodies to cellular proteins that interfere with the interpretation of the AGID and the indirect ELISA are not detected in the blocking ELISA (Anderson, 1984). None of these serological tests should be performed on a single sample to detect the presence of antibody; rather paired samples should be tested (Hoff et al., 1992).

Western immunoblots have been developed to identify BTV protein-specific antibodies and thus can be used to measure the immune response of animals to BTV infection. Such tests can also be used to identify antibody isotype (Adkison et al., 1987).

3. Antigen detection

The direct detection of BTV antigen in clinical samples provides a rapid means of diagnosis but is less sensitive than methods requiring replication of BTV virus (Calisher, 1992). Antigen capture ELISA techniques have been developed, using a horseradish peroxidase-antibody conjugate (El-Hussein et al., 1989a) or a biotin-avidin immunoperoxidase system (Mecham et al., 1990), for the detection of BTV viral proteins in the vector. In antigen capture (AGC) tests, diagnosis is effected by detection and simultaneous identification of virus specific antigens. Theoretically, this is the preferred technique for early diagnosis because results are not dependent upon the host responding immunologically to the virus. One antigen

capture antigen ELISA can detect $3.8 \log_{10}$ TCID/ml in BTV-infected cell culture lysates, and 2.3 to $4.8 \log_{10}$ TCID/ml of BT virus in BTV-infected midges (El-Hussein et al., 1989a). This technique is sensitive enough to detect a single infected midge in a pool with 99 noninfected midges. The antigen capture ELISA cannot detect BTV directly from infected blood, presumably because of low virus titer or the presence of antigen-antibody complexes. In addition, the presence of high antibody titers, antigen-antibody equivalence, immune complexes, and cross-reactions with closely-related viruses could all interfere with capture of the viral antigen in the EIA test, yielding false negative results. To enhance detection, virus can be amplified in cell culture and then detected by the ELISA from the cell culture supernatant (Mecham, 1993).

Many collection strategies for *C. variipennis* incorporate formalin, polyethylene glycol, or other fixatives to preserve the specimens. Such fixations often greatly complicate detection of protein antigens by destroying the specific antigenic determinants responsible for reactivity in the assay (Cornett et al., 1985). Storage of the *Culicoides* at -70°C for 2 months or more can be detrimental to the detection of the virus (Tabachnick and Mecham, 1991).

Other antigen-detection tests include fluorescent antibody (FA) tests that have been used with great success to detect BTV in tissue samples of *C. variipennis* (Jennings and Boorman, 1980; Ballinger et al., 1987) and immunoperoxidase tests to detect viral antigens in formalin-fixed BTV-infected tissues and paraffin sections (Cherrington et al., 1985). An avidin-biotin complex immunoperoxidase technique has been used successfully to identify BTV antigens in aborted bovine fetus brains (Anderson et al., 1989). Immunoperoxidase staining of BTV-infected CPAE cells on multiwell slides is more sensitive and rapid than visual inspection for cytopathic effects on infected cells or cDNA/RNA hybridization (Wechsler et al., 1990).

4. Nucleic acid detection

Direct detection of BTV-viral nucleic acid in infected tissue of the host or vector provides specific evidence of the presence of BTV. Traditional methods of nucleic acid detection include slot-blot, southern or northern blot and direct solution hybridization. Newer methods rely on nucleic acid amplification methods such as the polymerase chain reaction. Concerns with antigen-antibody equivalence, antigen clearance, latent infections or other infections without substantial antigen production, and specificity of

immunoglobulins are eliminated, while the sensitivity of the EIA is retained.

a. Nucleic acid hybridization

First described by Gillespie and Spiegelman (1965), nucleic acid hybridization is one of the standard research tools used by molecular biologists today. Hybridization with a labeled probe is one practical way of detecting complementary target sequences in a complex nucleic acid mixture (Wetmur, 1991). In these techniques, diagnosis is effected by detection of virus specific nucleic acid sequences by hybridization. Hybridization tests usually consist of 4 steps: sample preparation and denaturation, addition of labeled probe and hybridization, removal of unbound probe, and the detection of bound probe (Birkenmeyer and Mushahwar, 1991). Various types of hybridization methods have been used to detect BTV including: blot hybridization, *in situ* hybridization, and solution hybridization.

1). Blot hybridization

Blot hybridization is a technique in which target DNA or RNA is applied directly to a nitrocellulose or nylon-based membrane using a dot- or slot-blot apparatus, and the nucleic acids are linked to the membrane. A solution containing labeled probe is added, and the probe is allowed to hybridize with the bound nucleic acid. After eluting the

unbound probe, the hybrids are detected. The nucleic acid probes are labeled to high specific activity and provide a sensitive, specific, and reliable method for the diagnosis of virus infections.

A number of blot hybridization methods have been deployed to detect BTV utilizing various complementary DNA probes (Wilson, 1990). Diagnostic hybridization probes have been developed by molecular cloning of BTV genomic segments and subsequently used in dot-blot (Squire et al., 1985) and northern blot hybridizations (Unger et al., 1988). RNA/RNA hybridizations at low, medium and high stringencies provided an estimation of the percentage of homology among the dsRNA segments (Knudson et al., 1982; Kowalik and Li, 1987). The genes coding for NS1 and NS2 were the most conserved. Most of the RNA segments coding for the core proteins were also well-conserved while the outer capsid protein coding segments displayed a wide degree of sequence divergence. Segment 2 had little or no cross-hybridization among the five serotypes.

Hybridization with cloned BTV segments has revealed that segments 1, 3, 4, 6, 7, 9, and 10 are conserved between BTV serotypes, whereas segments 2, 5, and 7 can be variable (Dunn et al., 1989). The genetic relatedness between different BTV serotypes and strains from the same or different continents have been studied (de Mattos et al.,

1992). Segment 7 showed the most variation among serotypes, while segments 9 and 10 were highly conserved among all samples tested despite distant geographical origin. A genus-specific probe cloned from segment 10 hybridized to all five U.S. serotypes, but not to EHDV-1 and EHDV-2 (DeMattos et al., 1989a).

Molecular hybridization may be useful not only in the diagnosis of BTV infections but also in the simultaneous type specific identification of the infecting virus. Serotype-specific probes have been derived from segment 2 of BTV-17 and BTV-11 (deMattos et al., 1989b). In contrast, clones of segments 6, 8, and 9 are all genus-specific to varying degrees (Dunn et al., 1989). A radioactively-labeled probe derived from the genome segment that encodes NS1 was found to be very sensitive for the detection of BTV, because NS1 mRNA is transcribed more abundantly than the other target viral mRNA's (Venter et al., 1991). However, blot hybridization is less sensitive than immunofluorescence for detecting BTV in blood mononuclear cells from BTV-infected cattle (de la Concha-Bermejillo et al., 1992). Both methods are less sensitive than virus isolation.

2). Sandwich hybridization and solution hybridization

Sandwich hybridization utilizes two adjacent, non-overlapping probes: an analyte capture probe and a labeled

reporter probe. Sandwich and blot hybridization have comparable sensitivities when detecting BTV-infected midges (Schoepp et al., 1992). Solution hybridization utilizes complementary RNA probes in a liquid hybridization format followed by an affinity-based hybrid detection (Tenhunen et al., 1990). Solution hybridization can be adapted for use with crude biological samples without first purifying the mRNA. Hybridization occurs directly in guanidine thiocyanate solution, using the medium as both a cell lysing reagent and later, as a hybridization medium (Thompson and Gillespie, 1987; Olson et al., 1990). Solution hybridization has been used to detect BTV with respect to serogroup and serotype (Dangler et al., 1988). The hybridization can be performed rapidly (3 hours), with a sensitivity level of 10^4 PFU from a suspension with a concentration of 10^6 PFU/ml.

3). *In situ* hybridization

In situ hybridization has been investigated as a diagnostic procedure but may find wider application in pathogenesis studies (Venter et al., 1993). Infected cells or tissue sections are fixed on a glass slide and then the cells are exposed to a labeled probe under hybridization conditions (Squire et al., 1987). This method is not as sensitive as virus isolation or antigen detection when assaying for most BTV serotypes (Schoepp et al., 1991). No

infected blood mononuclear cells could be observed from an experimentally-infected heifer using an *in situ* hybridization procedure that had previously detected BTV in infected cultured bovine mononuclear cells (Dangler et al., 1990b).

4). Hybridization probes

Radiolabeled hybridization probes have been widely used to detect a variety of virus genomes in clinical specimens using mixed-phase hybridizations. Although radioactively-labeled probes have proven to be exquisitely sensitive and specific, there are problems associated with radioisotopes, including: 1) short half-life of many radioisotopes, 2) expense of radiolabeled compounds, 3) safety of personnel, and 4) waste disposal. A further problem is the time necessary for autoradiography to detect the formation of hybrids. Thus, there is a need for an alternate non-radioactive technique to test for virus genome sequences in specimens.

The biotin-avidin/streptavidin system was one of the first "non-isotopic" systems to be developed. Although sensitive, the signal-to-noise ratio was often poor because of high background (Martin et al., 1990). The GeniusTM nonradioactive nucleic acid labeling and detection system uses a digoxigenin-labeled nucleic acid probe for hybridization; hybrids are detected with an immunoassay

using an anti-digoxigenin antibody conjugate (Martin et al., 1990). A digoxigenin-labeled RNA probe cloned from an NS1 segment of BTV-17 could detect all 24 BTV serotypes in infected cell cultures (Brown et al., 1993).

b. Polymerase chain reaction

Of the basic molecular techniques that have been developed in the last decade, none have had a greater impact on disease diagnosis than the polymerase chain reaction (PCR) (Pershing, 1991). PCR is essentially a method for amplifying nucleic acids. In the PCR technique, the analyte sequences are denatured and two oligonucleotide primers are annealed to complementary strands on either side of the analyte sequences. These primers are then extended by a DNA polymerase. The new double-stranded analytes are denatured, the primers annealed, and then extended by the polymerase. The amount of analyte is increased geometrically with each cycle and a single copy of DNA can be increased up to 1,000,000 copies after 20 cycles. Saiki et al. (1988), described the first use of *Taq* DNA polymerase, a thermostable DNA polymerase derived from the *Thermus aquaticus*, in place of the Klenow fragment of *E. coli* DNA polymerase I, which significantly improved the sensitivity, specificity and yield of the products being amplified. After amplification, analyte sequences can be detected using a labeled probe specific for a portion of the amplified

sequence or by ethidium-bromide (Et-Br) staining of size-specific amplified products.

PCR is being applied in molecular biology laboratories around the world for use in diagnostics, sequencing, cloning, and population genetics. However, for the PCR technique to be applied for arbovirus diagnosis, several considerations need to be addressed. Nearly all of the arboviruses have RNA genomes; therefore, reverse transcriptase is required to make the initial cDNA copy of the viral gene or genome. Sequence information must be available to synthesize the oligonucleotide primers. PCR can be used not only to detect viruses but also to type them as seen with the assay developed to detect dengue viruses (Lanciotti et al., 1992).

PCR has been adapted for the detection of BTV in several laboratories (Dangler et al., 1990a; Wade-Evans et al., 1990; Akita et al., 1992; Wilson and Chase, 1993). Using PCR, BTV has been detected in infected red blood cell fractions and the buffy coat, but not in whole blood samples from sheep (McColl and Gould, 1991). However, problems with the reverse transcriptase step, recovering the RNA from the sample, and blood-binding interfering molecules have been described for PCR-based diagnostics (Dangler et al., 1990a). The PCR sensitivity has recently been increased in clinical

samples such as semen by combining PCR with the use of non-radioactive chemiluminescent hybridization (Akita et al., 1993).

c. Reversible target capture assay

Although the potential for diagnosis of virus infections using nucleic acid hybridization is manifest, current hybridization formats have serious limitations for clinical use. Blot hybridization assays have slow reaction kinetics and low sensitivities for clinical samples. Blot hybridizations often require tedious sample preparation protocols, and radiolabeled probes are needed for acceptable levels of sensitivity. Also, this format is not easily adapted for processing large numbers of specimens in a timely fashion. Ideally, any hybridization assay to be used routinely in clinical laboratories should be as technically simple and as sensitive as EIA.

Extensive RNA extraction procedures using caustic materials cause problems that could be overcome by using a reversible target capture (RTC) system, in which RNA is captured from crude cell lysate (Thompson et al, 1989a, 1989b). The target nucleic acid is hybridized in solution with a dA-tailed capture probe and a labeled probe (Collins and Hunsaker, 1985). Magnetic beads containing oligo (dT) capture this hybridization complex. After washing away unhybridized probe, the complex is eluted and captured on

fresh beads. A detection limit of 0.2 amol (10^{-18} mol) of target using ^{32}P as the radioactive label has been attained (Morrissey et al., 1989b). Adding a fourth capture and using a non-radioactive probe such as biotin, 0.25 amol of target could be detected in less than an hour (Hunsaker et al., 1989). The RTC technique has been successfully adapted for the detection of dengue-2 viral RNA (Chandler et al., 1993).

The reversible target capture hybridization assay has many advantages over PCR, including a short reaction time (3 hours compared to 12 hours), no requirement for a reverse transcriptase step, no amplification of contaminating sequences, and no need for an extensive RNA extraction step before undertaking the reaction. The sensitivity and specificity of the RTC assay is predicated in large part upon the initial separation of the analyte from contaminants in the clinical specimen. Such contaminants are major causes of nonspecific and false negative results in PCR based diagnostics. Combination of components of the RTC system to purify the analyte and subsequent PCR amplification of the target could theoretically provide the most sensitive and specific technique for BTB surveillance.

H. Control of BTV Infections

BTV is one of the most feared diseases in sheep-producing countries--many people feel that once BTV has entered a country, there is little hope in eliminating it (Roberts et al., 1992). Eradication of BTV is unlikely once BTV becomes enzootic (Holbrook et al., 1985). However, integrated control measures can drastically reduce the spread of BTV. These measures include preventive control such as vaccine use and environmental control affecting both the vector and the vertebrate host.

1. Vaccines

The first polyvalent vaccine was prepared from the Theiler strain (BTV-4), BTV-3, and BTV-12 (Gorman et al., 1983). Despite its widespread use, the vaccine occasionally produced severe reactions and in other cases it failed to confer full protective immunity (McKercher et al., 1957). Current vaccine procedures use modified live virus preparations, and can include 15 or more different serotypes. These induce protective immunity, but can infect a fetus when administered to pregnant ewes and cause teratogenic effects such as fetal encephalopathy (Shultz and Delay, 1955). Unfortunately, *C. variipennis* can become infected with and biologically transmit the vaccine virus, which could result in new foci of infection in BTV-free areas (Foster et al., 1968). This type of vaccine also has

the potential for genetic reassortment with a wild-type virus and thus reverting to virulence (Walton, 1992). Combining multiple serotypes in the same vaccine may facilitate recombination between the segmented genomes with the possible emergence of new and potentially more virulent serotypes (Strating, 1985). A simpler approach would be to serially inoculate 2 or 3 monovalent preparations separated by a 3-week period to give rise to a broad serotypic response; however, this is hardly feasible (Jeggo et al., 1983). In the U.S., a modified live BTV-10 vaccine is the only product licensed for use in sheep; it is not available for use in cattle (Walton, 1980). Unfortunately, the most frequently isolated serotypes have been BTV-11 and BTV-17, (Barber, 1979), thus this vaccine may be ineffective in many areas.

Other attenuated, subunit, and inactivated vaccines are also being investigated. Purified VP2 has been used as a single protein subunit vaccine inducing neutralizing and hemagglutination-inhibiting antibodies (Huismans et al., 1987d). Recombinant DNA technology could be used to synthesize VP2 on a large scale by molecular cloning in an alternate host (Huismans, 1985). However, it is unlikely that a VP2 subunit vaccine will ever replace the attenuated live virus vaccine. A large amount of purified VP2 is needed for even one single protective dose, and the

neutralizing antibodies that are induced are serotype-specific (Huisman et al., 1987d). Another vaccine approach has utilized gamma irradiation, which can inactivate BTV by disrupting the internal molecular structures but cause little harm to the surface proteins (Campbell et al., 1985).

BTV-like particles derived from multiple baculovirus expression vectors have been used to inoculate sheep and have been shown to be highly immunogenic with the appropriate adjuvant (Roy et al., 1992). The virus-like particles containing VP3 and VP7 were more immunogenic when combined with outer capsid proteins such as VP2 (Roy and Erasmus, 1992).

Some studies have indicated that sheep exposed to BTV, such as with a vaccine, demonstrate immune enhancement and may have more severe clinical disease than nonexposed sheep upon subsequent challenge exposure (Stott et al., 1985b; Luedke, 1969). Vaccination of sheep with inactivated BTV did not result in the production of a hypersensitivity reaction upon subsequent exposure to virulent BTV (Mahrt and Osburn, 1986). However, these sheep did not produce detectable neutralizing antibodies and they were not protected against subsequent exposure to the virus.

2. Vector source reduction

Vector source reduction or vector control often does not require the eradication of a vector but rather the

significant reduction of their numbers. Control techniques include chemicals, habitat management and biological agents (Mullens, 1992). Insecticide, a chemical control, can be applied to the environment at seven day intervals to eliminate infected *Culicoides* females. External and systemic treatment of ruminants with insecticide may reduce the spread of disease. Habitat management includes water level fluctuation, adjusting organic input, and better drainage. Water fluctuations at 1 week intervals drastically reduce *C. variipennis* production (Mullens and Rodriguez, 1989). Ponds that are deeper and have steeper slopes support lower densities of *C. variipennis* (Mullens, 1989). Biological control through the use of a nematode that parasitizes *C. variipennis*, may be used in the future (Mullens, 1992). Another method of control is the sterile-male technique, which has been successful in eradicating the screw-worm fly from the U.S. However, *Culicoides* males artificially sterilized with gamma radiation tend to recover fertility at later matings (Jones, 1967).

3. Vertebrate host control

Host manipulation can be achieved such as housing vertebrate animals overnight to prevent vector biting activity or moving vertebrate animals to a higher altitude. To control BTV infection in a vertebrate herd it may be necessary to eliminate BTV-infected animals, especially

severely clinically affected sheep. Prohibiting the movement of BTV-infected ruminant animals has only a very limited effect on the overall spread of the disease but should be applied after an outbreak (Webster et al., 1992).

I. Research goals

The first research goal for the following studies was to investigate the possibility that vertical transmission of BTV in the insect vector acts as the overwintering mechanism of BTV in temperate regions. Extensive prospective field studies were conducted in Colorado in four BTV endemic areas in 1992. Annual BTV activity has been documented in these areas without a known mechanism of transeasonality. Preliminary investigations in these areas in 1990 resulted in the detection of BTV in *C. variipennis* adults collected in the middle of June. This early detection was noteworthy; it had never been reported before so early in the season. Therefore, a study was designed to test both the vector and the vertebrate hosts in these areas to try to delineate the mechanism of overwintering. Larval stage *Culicoides* were collected throughout the transmission season in both 1991 and 1992. The larvae were processed for BTV analyte by both conventional means such as virus isolation in cell culture, and by utilizing newly-developed diagnostic techniques such as RNA blot hybridization and capture antigen ELISA techniques. A variety of detection techniques were utilized

because there was little information on the efficacy of these tests in field trials, especially for the testing of larval specimens. Adult *C. variipennis* collected in the feeding activity study (Chapter III) were assayed by the capture-antigen ELISA technique to survey for BTV prevalence in these areas. Sentinel calves and sheep were placed at two of the farm sites and were monitored, along with herd animals at all four sites, for BTV seroconversion. Detection of virus analyte in the larvae by one or more of the diagnostic techniques would suggest that vertical transmission of BTV occurs in the vector.

The second research goal was to study the host preferences and temporal feeding activities of *C. variipennis* in Colorado. These activities have not been well-characterized in these areas and would be important determinants in future vector control strategies. Intensive prospective studies were conducted on two of the farm sites studied in the previous chapter. The experimental design utilized a drop trap method in which insects were attracted to bait animals for a period of time, then a frame-supported net was lowered over the animal and the insects were collected by vacuum aspiration. The study design consisted of a team of investigators, a sentinel calf and a sentinel sheep at each farm site. During the summer of 1992, the traps were operated twice a week, for three (Ausman site) or

four (Fields site) months. Each trap night started at thirty minutes before sunset and each trapping cycle--consisting of exposure of animals to insects, lowering of traps, and aspiration of insects--lasted for thirty minutes. There were five trapping cycles each night. After collection, the insects were identified and the *Culicoides* were tested using the capture antigen ELISA for BTV. This allowed determination of the prevalence of BTV in the adult *Culicoides* attracted to the sentinel animals.

During the previous studies, new research protocols and techniques were being developed. The third research goal involved an identification problem observed in the first study. *Culicoides* larvae could be collected, sorted and then tested for BTV, but identification of the larvae to the species level is very complex, requiring a large time commitment to morphologically identify just one or two larvae. In the study, we tested thousands of larvae, without identifying them to the species level, assuming that many were *C. variipennis*. We then decided to develop a polymerase chain reaction using tRNA gene primers to differentiate *Culicoides* at the species level. Each *Culicoides* species would have a characteristic "fingerprint" banding pattern to differentiate them from other species. This technique would work on adult as well as larval stages of *Culicoides*.

Another problem seen in the field studies was the low sensitivity and lack of concordance with the detection methods used to detect BTV. For the fourth research goal, a reversible target capture (RTC) assay was developed to detect BTV in both infected cell culture and infected *Culicoides*. Because of low sensitivity, the RTC was combined with the polymerase chain reaction to increase the sensitivity of the assay.

The following studies describe the molecular epidemiology, transeasonality, and taxonomy experiments conducted with BTV and *Culicoides variipennis*.

II. TRANSEASONALITY OF BTV

A. Introduction

In many parts of the world, *Culicoides* have specific periods of inactivity each year due to adverse environmental conditions. Mechanisms that promote survival of BTV during these periods of adverse climatic conditions remain to be elucidated. Determination of the overwintering or transeasonality mechanisms may lead to more effective control measures for this important veterinary pathogen.

Preliminary investigations on BTV transeasonality in Colorado may be noteworthy in this regard. From June to July 1990, adult *Culicoides* were obtained from one of the breeding sites investigated in this study. *Culicoides* were collected in light traps, frozen, sorted into pools of 50, and tested for presence of virus using standard virus isolation techniques and an enzyme-linked immunosorbent assay. Four field-collected pools were tested for each collection date. Sample pools from 6/12/90, 6/14/90, 6/21/90, 6/26/90, and 6/27/90, tested positive by virus isolation and indirect fluorescence tests and these were confirmed with the antigen detection-ELISA assay. Detection of BTV in vectors this early in the season is noteworthy.

Admittedly, these adults could have been infected by feeding on viremic vertebrate hosts, but it is also possible that adult flies could have been infected through vertical transmission.

Until recently, diagnostic methods for the detection of BTV were insensitive, especially for detection of unpassaged field isolates. New sensitive and specific molecular techniques now permit detection of BTV, even in very low titer. Molecular techniques also permit detection of viral nucleic acid and antigen in the absence of detectable infectious virus. Such techniques may be useful in the determination of mechanisms of transeasonality of BTV.

Isolation of BTV from field-collected *Culicoides* is difficult for many reasons. A potential pitfall is the difficulty in isolation of virus from pools of vectors. Titers of 1000 to 10,000 plaque forming units (PFU) of Turlock and Hart Park viruses were reduced to undetectable levels when virus was triturated with 25 or more uninfected larvae of *Culex tarsalis* prior to isolation (Ksiazek et al., 1985). Suspensions of less than 25 larvae had little adverse effect on virus isolation. Suspensions of increasing numbers of normal adult mosquitoes have been shown to interfere with the detection of arboviruses, resulting in decreased numbers of viral plaques detected (Yuill, 1969). This mechanism of plaque reduction by

mosquito suspensions is not understood but may be accounted for on the basis of virus weakly bound to small particles of the mosquito suspension that tend to aggregate in increasingly concentrated mosquito suspensions. In addition, *Culicoides* larvae have been difficult to process in the past due to excessive mortality, especially when collected from a field environment and transferred to a laboratory environment. Excess mortality also occurs if the larvae are not processed expeditiously and remain in the larval pans too long. Since BTV may be inactivated in infected *Culicoides* that die in traps or during collection, adult specimens must be collected early in the morning to preclude dessication of specimens. Field-collected BTV is often difficult to isolate, requiring passage in chicken embryos to adapt the virus to produce detectable pathogenic effects.

However, in these instances, molecular techniques should yield positive results in the absence of infectious virus as detectable by conventional means. Viral antigen and viral genomic RNA would theoretically be detectable, even if the virus is inactivated or transcriptionally latent (El Hussein et al., 1989a; Schoepp et al., 1990). This is a principal advantage of using molecular techniques for arbovirus surveillance in vectors.

A previous study in these breeding site areas in northeastern Colorado investigated the biology and

population dynamics of *C. variipennis* on a seasonal basis. Barnard and Jones (1980b) found that *C. variipennis* larvae were most abundant in a zone extending from 7 cm above to 30 cm below the shoreline, as well as 5 cm deep in the mud. Overwintering larvae escaped freezing by moving deep into the mud under deep water. Adults emerged from March until late October from larvae that overwintered at the 4th instar stage. Third and 4th instars were observed in November and December, but only 4th instars were recovered in March. First instar larvae appeared in April with larval densities the highest from June until September. Pupae were only found on mud at or above the shoreline from April through October. The numbers of larvae collected were greater at dawn than during the rest of the day. The life cycle was approximately 2 weeks for *C. variipennis* during the summer, which is consistent with the life cycle seen under optimal laboratory conditions (Barnard and Jones, 1980b).

In this study, extensive prospective field trials were conducted in Colorado in BTV endemic areas. These areas have been studied for the past 10 years by scientists from the USDA/ARS Arthropod-borne Animal Disease Research Laboratory (ABADRL) in Laramie, Wyoming, and annual BTV activity has been documented without a known mechanism of transeasonality. Farmers in these areas supported and permitted intensive surveillance for BTV-infected

vertebrates and BTV-infected larval and adult-stage *Culicoides* vectors on their premises. To determine the overwintering mechanism of BTV, both adult and larval *Culicoides* were collected in a predetermined schedule throughout the transmission season and processed for BTV analyte. The rationale for the study design was as follows. Detection of virus analyte in larvae before the seasonal emergence of adults would strongly suggest that BTV overwinters in the vector. Lack of detection of BTV analyte in field collected larvae, and subsequent detection of virus in adults would implicate vertebrate hosts in overwintering. If BTV was not detected in the larvae and only became detectable in the adult flies after seroconversions in sentinel bovines placed in the herds, then vertical transmission in the vector would seem to be an unlikely overwintering mechanism. This study design also permitted an assessment of the comparative diagnostic efficacy of various assays for detection of unpassaged BTV isolates from vectors. Antigen detection, nucleic acid hybridization (blot, reverse target capture, and PCR), and virus isolation (in various cell culture systems) were to be compared for sensitivity and specificity for detection of BTV in vectors.

The research hypothesis for this study was that vertical transmission of BTV in the *Culicoides* vector was

the overwintering mechanism in temperate regions such as Colorado. The specific aims of this study were to collect larvae from breeding sites in BTV-endemic areas in Colorado and utilize newly-developed diagnostic techniques such as molecular hybridization and antigen detection, as well as virus isolation, to detect BTV. Sentinel animals, as well as herd animals, were monitored for BTV seroconversion at these study sites.

B. Materials and Methods

1. Field study sites

Four premises in Colorado were closely monitored during the study period; all contain permanent breeding sites for *C. variipennis* (Figure 2.1). The four farms were the Ausman, Howard and Wagner farms near Brighton, Colorado (Figure 2.2) and the Fields farm near Loveland, Colorado (Figure 2.3). Annual BTV activity had been demonstrated by virus isolation or seroconversion on each of these premises for many years by USDA/ARS-ABADRL scientists as part of their ongoing field surveillance and control program. The Ausman farm (Figure 2.4A) was strictly a dairy operation consisting of approximately 120 milking Holstein cows, 50 Holstein unbred heifers, 6 veal calves, 3 horses, and 2 dogs. Many small mammals such as shrews, voles, and mice were observed in the feed areas of the farm. The Howard

farm (Figure 2.4B) was a beef/hay/silage operation with 200 cow-calf Hereford/Angus cross units, 12 horses, 1 dog, and many chickens, ducks, and geese raised in a small hatchery on the farm. The Wagner farm (Figure 2.4C) was strictly a dairy operation containing 140 Holstein cows, assorted Holstein calves, 1 Holstein bull, 4 dogs, and a few cats. The dairy herd has been a documented closed herd since 1945. The Fields farm (Figure 2.4D) was a beef/show calf operation with approximately 7 show calves (Friesian, Amerifax, and Limousine), 8 Hereford/Angus cow-calf units, 3 horses, 10 pigs, 2 sheep, various cats, dogs, and raccoons, exotic waterfowl and a nearby large prairie dog farm. All farm sites had areas of pasture with poor drainage contaminated with various waste and sewage effluent (Figure 2.5).

2. Collection of *Culicoides* at the premises

A prospective cross-sectional sampling technique was used to collect random samples from the population-at-risk (*C. variipennis*). Adults were collected weekly at the four established breeding sites from March to October and larvae collected every third week all year round at three of the breeding sites. A total of 4 to 5 samples were collected at each breeding site (Figures 2.6A-E). These collections were then sorted into pools of 25-50 and numbered according to pool number and sample area. A minimum of twelve pools from each collection date (three pools from each sample area)

were to be collected. It was determined that, at the end of one year, almost 600 *Culicoides* pools or 12,000 *Culicoides* larvae would need to be tested. This sample size was based on the expected frequency of BTV infection in *C. variipennis* (0.1%), human resources and money, and statistical accuracy (99.5%) (Leech and Sellers, 1979). If BTV infected 0.1% of the field *Culicoides* population, then 600 pools total would have to be sampled in a year to get an accurate representation if BTV infection were occurring through vertical transmission.

During the study, approximately 62,000 larvae were collected and tested during the two-year period. This number almost tripled the minimum annual sample size needed for the study.

a. Larval collection

Larvae were collected from poor drainage areas in the pastures, which contain large amounts of organic material. Three samples were taken at each of the 4 to 5 sampling sites at each breeding site: 1) The water line of the waste effluent, 2) Mud under the water at a depth of two centimeters, and 3) Water taken from shallow areas without disturbing the mud underneath (Figure 2.7). Sampling areas, sites and farm sites are listed in Table 2.1 with the corresponding pool letter designation used to identify each pool in the study. Larvae were collected by filtration of

the mud samples, sorted into pools of 25 (1991) or 50 (1992), and then frozen at -70°C in phosphate buffered saline (PBS) with 10% fetal bovine serum (FBS). This increase in pool size from 25 to 50 was due to the increased number of total larvae that needed to be processed and the time constraints placed on processing these larvae.

b. Adult collection

Adults were captured alive overnight using CDC light traps at the waste effluent sites near the breeding sites (Figure 2.8). Adults were collected into a linen net bag during the night, returned alive to the laboratory, and frozen at -70°C . Adults were sorted and identified to the species level on a chill table using a binocular stereoscope.

c. Negative and positive controls

Negative control pools used in each test were comprised of larvae from the AK colony maintained at the USDA-ABADRL in Laramie, WY. This colony originated from field material collected in 1973 and is certified to be BTV-free. BTV-positive control pools were made up of negative larvae combined with BTV-infected viral cell culture lysates to yield final titers of approximately $2.5 \log_{10}\text{TCID}_{50}$ (El Hussein et al., 1989). Negative adult *Culicoides* were obtained from the same colony as the negative larvae. Positive adult *Culicoides* were obtained by intrathoracic

inoculation of BTV-17, and flies were maintained for 14 days at 26°C and 40% relative humidity. After 14 days, the flies were frozen and stored at -70°C.

3. Sentinel animals

a. Sentinel calves

Four, 4-month old Holstein dairy heifers were acquired (Figure 2.9A). After demonstration that each was BTV-antibody free, one was placed at each farm site. Three were allowed to mix freely with each herd. The calf at the Ausman site was placed in a pen next to the *Culicoides* breeding site and the pasture containing the milking cows.

b. Sentinel sheep

Two, 6-month old Dorset ewes were acquired and demonstrated to be BTV-antibody free (Figure 2.9B). One was placed in the same pen as the sentinel calf at the Ausman farm and the other was placed in a separate pen at the Fields farm, adjacent to the pen containing the sentinel calf.

c. Herd cattle

Thirty-six calves (<2 years of age) were bled on 5/19/92 from the Howard and Wagner farm sites. Sixty-nine herd calves (<2 years of age) were bled on 11/10/92 from all four farm sites. These calves included those bled in the spring.

d. Serum samples

All animal blood samples were tested for seroconversion by agar gel immunodiffusion (AGID) and a competitive ELISA (cELISA) to establish a baseline prevalence rate. Ten ml of whole blood were collected from the jugular vein with a 2.5 cm 20-gauge needle into a heparin-treated vacutainer. Blood was refrigerated immediately, and after 24 hours, the samples were centrifuged at 1500 rpm for 10 min to separate cells from the serum. Samples of blood were collected monthly from the sentinel calves and ewes to monitor for seroconversion. They were also observed by the farmer and field workers for signs of ill health. After the study, the animals were donated to the respective farmers as compensation for their assistance with the study.

4. Vector diagnostic assays

Frozen larval pool aliquots were thawed in a 37°C water bath and the PBS/10% FBS solution was removed. Each larval pool was triturated in a cold (4°C), sterile mortar and pestle with 1 ml of fresh sterile PBS/10% FBS solution. This solution also contained 5 ug/ml of Fungizone (Amphotericin B), 100 units/ml of Penicillin and 10 ug/ml streptomycin. After trituration, the suspension was sonicated on ice for 20 sec at 70% power (Fisher Sonic Dismembrator, Fisher Scientific, Springfield New Jersey). This suspension was first stored at 4°C until all diagnostic

assays were performed and then at -70°C for long-term storage. A flow diagram of the vector diagnostic assay used in this study is shown in Figure 2.10. This diagram depicts the order in which the various assays were performed and the volume of sample needed for each assay. The vector diagnostic assays included: virus isolation on three types of cell culture lines (BHK-21, Vero, and C6/36), with subsequent fluorescent antibody detection and blot hybridization detection; direct nucleic acid blot hybridization; capture antigen enzyme-linked immunosorbent assay (ELISA) detection; and reverse transcription-polymerase chain reaction (RT-PCR).

a. Virus isolation (VI)

Two vertebrate cell lines, Vero (green monkey kidney), and BHK-21 (baby hamster kidney), and one invertebrate cell line, C6/36 (*Aedes albopictus*), were used. Cells were grown to monolayer confluency in 24-well culture plates. The plates were sealed and incubated at 37°C for the vertebrate cell lines and 25°C for the invertebrate cell line. Fifty μl of each pool was inoculated onto each cell culture well after first removing the medium. After inoculation of the cell culture monolayer, the specimen was allowed to absorb with rocking for 15 min. Care was taken not to allow the cell monolayers to dry. Fresh medium (0.5ml) was then added and the plates were sealed and incubated. BHK-21 and Vero

cells were incubated at 34°C for 3 and 5 days, respectively. C6/36 cells were incubated at 27°C for 4 days. Each sample was passaged three times by scraping the cell monolayer with a pipet tip and transferring both the cells and the supernatant to a new 24-well plate with an 80-90% monolayer of cells. After 3 passages, the cells were fixed on slides using acetone (4°C) for assay by an indirect fluorescent-antibody test, and also tested by RNA blot hybridization.

b. Indirect fluorescent-antibody test (IFA)

The slides were stained with a 1:200 dilution of anti-BTV mouse IgG antibody, and incubated for 1 hour at 37°C. This antibody was a monoclonal derived from cell culture supernatant fluid from a hybridoma cell line 10A1.34 and was probably specific for NS1 (R. Schoepp, CSU Dissertation, 1989). The slides were washed 3 times in PBS, air-dried, and a 1:200 dilution of the fluorescein-conjugated, goat anti-mouse IgG was applied to each slide. After incubation at 37°C for 1 hour, the slides again were washed 3 times in PBS, allowed to air dry, and examined with an epifluorescence microscope. Samples with fluorescence above background levels were considered to be positive.

c. RNA blot hybridization

1. RNA extraction

Total RNA was extracted from each larval pool by the single-step acid guanidinium thiocyanate method (Chomczynski

and Sacchi, 1987). Briefly, 200 ul of the triturated larval suspension was added to 200 ul of the denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol) and vortexed for 20 sec. Then 20 ul 2 M sodium acetate, pH 4.0, 200 ul water-saturated phenol, and 40 ul chloroform-isoamyl alcohol (49:1) were added sequentially, mixing by inversion after each addition. The tube was then vortexed for 10 sec and incubated for 15 min on ice. After the tubes were centrifuged at 10,000 rpm (12,100 X g) for 30 min at 4°C, the aqueous phase containing the RNA was transferred to a fresh tube, mixed with 200 ul of isopropanol, and placed at -20°C overnight to precipitate the RNA. After a second centrifugation at 10,000 rpm (12,100 X g) for 30 min at 4°C, the resulting RNA pellet was resuspended in 75% ethanol, pelleted, air-dried, and dissolved in 100 ul 0.5% DEPC-treated water.

RNA extraction was also performed on cell cultures after three passages. After the third pass, 200 ul of the cell and supernatant mixture was added to 200 ul of the denaturing solution and the procedure described above was followed.

2. Blotting procedure

Extracted RNA was denatured in TE buffer, pH 7.0 (10 mM Tris-HCl, pH 7.0; 1 mM EDTA pH 8.0), for 5 min at 100°C and

then immediately placed on ice. An equal volume (100 μ l) of 2 X dilution buffer (1 X dilution buffer is 7% formaldehyde, 6 X sodium citrate, sodium chloride (SSC) was added to prevent renaturation of the double-stranded RNA. Nylon membrane (Nytran, pore size 0.45 μ m, Schleicher and Schuell), was prewetted with sterile water, rinsed in 6 X SSC, then assembled in a Minifold II slot-blot apparatus (Schleicher and Schuell) with two sheets of blotting paper wetted in 6 X SSC. Approximately 0.5 ml of 6 X SSC was applied to each slot and drawn through the membrane under low vacuum pressure. An aliquot of each sample (100 μ l) was applied to its designated slot under low vacuum pressure. The blots were then exposed to a U.V. light source (160 J/m²) at a distance of 15 cm for 3 min, and then baked at 80°C for two hours to cross-link the RNA to the filter. Blots were then sealed in plastic hybridization bags and were then hybridized or stored at 4°C.

3. Labeling of probes and signal detection

Previously, a cDNA of the L3 genome segment of BTV-17 was cloned into pBR322 and kindly provided by Dr. Polly Roy, Birmingham, Alabama. This plasmid was designated pBTV17-3. A 674 bp fragment of pBTV17-3 was subcloned into the BamHI site of pGEM-1 plasmid and designated pRSG1.674 (Schoepp et al., 1990) (this plasmid will be discussed in greater detail in Chapter 5 section B.3). When utilized as a probe, this

plasmid hybridizes with all five U.S. BTV serotypes and fourteen other BTV serotypes, but does not hybridize to EHDV (R. Schoepp, CSU Dissertation, 1989).

In these studies, probes were labeled radioactively for testing of 1991-collected larval pools, and labeled nonradioactively for testing of 1992-collected larval pools. Radioactive probes were prepared by transcription of RNA from plasmid pRSG1.674 with bacteriophage T7 polymerase (Promega) and ^{32}P -UTP (Amersham), using a Riboprobe labeling kit (Promega) following the manufacturer's instructions. The transcription template consisted of 1 ug linearized (HindIII) pRSG1.674 DNA (absorbance measured by spectrophotometry). Probe was separated from unincorporated nucleotides using a Sephadex G-50 spin column. The activities of the probes were determined by scintillation counting of trichloroacetic acid precipitates. Blots were pre-hybridized at 42°C for 2 hours in hybridization solution (50% deionized formamide, 6 X SSC, 5 X Denhardt solution, 0.1% SDS, and 200 ug/ml each of denatured salmon sperm DNA and tRNA. Fresh hybridization solution was added containing the prehybridization solution and 1 to 2 X 10^6 cpm/ml labeled probe, and hybridization carried out at 42°C for 24 hours in a shaking water bath. Post-hybridization washes consisted of four 5-min washes at room temperature with 2 X SSC, 0.1% SDS, two 25-min washes at 50°C with 0.1 X SSC,

0.1% SDS, two 5-min washes at room temperature with 2 X SSC, one 10-min wash at room temperature with 2 X SSC containing 10 ug/ml RNase A, and two 5-min washes at room temperature with 2 X SSC. The blots were dried and were exposed to X-ray film for 1-3 days. The films were developed manually according to standard procedure. Samples were considered positive if the intensity of the hybridization signal exceeded negative control background levels.

Nonradioactive detector probes were prepared by transcription of RNA from plasmid pRSG1.674 with digoxigenin-UTP using the Genius System RNA labeling kit (Boehringer Mannheim) and bacteriophage T7 RNA polymerase following the manufacturer's instructions. The transcription template consisted of 1 ug linearized (HindIII) pRSG1.674 DNA. The labeled RNA was precipitated with LiCl (4M) and ethanol, centrifuged, dried under vacuum, and resuspended in 100 ul diethyl pyrocarbonate (DEPC)-treated water. Activity of these probes was also determined by the addition of ³²-UTP as a tracer in the reaction which could then be precipitated with trichloroacetic acid. For detection of labeled probe, blots were washed in buffer 1 (150 mM NaCl, 100 mM Tris-HCl, [pH 7.5]) for 5 min and then incubated in buffer 2 (2% [wt/vol] blocking reagent dissolved in buffer 1) for 3 hours at room temperature. Blots were then transferred immediately to a fresh solution

of buffer 2 containing 150 mU of sheep anti-DIG antibody per ml conjugated to alkaline phosphatase for 30 min at room temperature. After washing twice for 15 min each in buffer 1 at room temperature, the blots were then washed once in buffer 3 (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 50 mM $MgCl_2$) for 2 min at room temperature. Blots were immediately covered with a thin layer of Lumi-Phos (Boehringer Mannheim) chemiluminescent substrate and sealed into a clear plastic bag. The blots were exposed to X-ray film for 5 to 30 min. The films were developed manually according to standard procedures. Samples were considered positive if the intensity of the hybridization signal exceeded negative control background levels.

Problems involving RNase activity were anticipated in detecting the single-stranded RNA. Special precautions taken to minimize RNase activity included: use of sterile disposable plasticware for RNA preparation; preparation of glassware by baking at 250°C for 4 h, followed by treatment with 0.1% diethylpyrocarbonate and autoclaving; wearing rubber gloves during preparation of reagents; procurement and use of chemicals, glassware, spatulas, restricted for RNA work; and where possible, treatment of buffers and solutions with diethylpyrocarbonate or RNAsin.

d. Antigen-capture EIA

Purified guinea pig-immunoglobulin G antibody was diluted in carbonate-bicarbonate buffer and coated on individual wells of 96-well plates at a concentration of 500ng of protein per well (El Hussein et al., 1989). This antibody is specific for the BTV serogroup and does not cross-react with other closely related Orbiviruses (El Hussein, CSU Dissertation, 1989). After incubation overnight at room temperature, the wells were washed 5 times with phosphate-buffered saline (PBS) containing 0.05% Tween 20. Pool samples (50 ul each) were aliquoted into each well, incubated for 1 hour at 37°C and washed 5 times in the PBS-Tween-20 solution. Anti-BTV-10 mouse ascitic fluid diluted 1:5000 in PBS-4% fetal bovine serum was added to each well (50 ul each), incubated for 1 hour at 37°C and washed 5 times in the PBS-Tween-20 solution. Goat anti-mouse antibody conjugated to horseradish peroxidase and diluted 1:10,000 in PBS-4% fetal bovine serum was added to each well (50 ul each), incubated for 1 hour at 37°C and washed 5 times in the PBS-Tween-20 solution. Substrate TMB (3,3',5,5'-tetramethylbenzidine) was added (100 ul in each well) and incubated for 10 min after which 50 ul of 2N sulfuric acid was added. A positive sample produced a bright yellow color. Absorbance was measured at 450 nm in a microplate reader (Biorad). Each sample was tested in

triplicate. Samples were positive if the absorbance value was greater than the mean value of the negative control plus three standard deviations.

5. Vertebrate diagnostic assays--serology

Sentinel and herd animals were monitored by the competitive ELISA (cELISA) and agar-gel immunodiffusion (AGID) tests.

a. Competitive ELISA (cELISA)

In the cELISA, BTV-specific antibody that may be present in the test serum competes with a BTV-specific monoclonal antibody component of the kit for attachment to BTV viral antigen which is coated to the surface of the plastic wells. Four replicates were tested for each sample, and positive and negative controls were included. The direct EIA kit was a gift from Dr. Michael Jochim of Veterinary Diagnostics, Inc. (Colorado). The test is specific for group-specific antibody to BTV, does not react with antibody to EHDV, and is reported to be equal in sensitivity to serum neutralization tests. The test was conducted following manufacturer's directions. Briefly, the test serum and monoclonal antibody were added simultaneously to the coated wells of the microtiter plate and incubated for two hours at room temperature. After washing the wells, anti-mouse IgG labeled with horseradish peroxidase was added and incubated for one hour at room temperature. Hydrogen

peroxide and the chromagen, o-phenylenediamine dihydrochloride (OPD), were then added and the development of a color reaction indicated the absence, or a very low level, of BTV specific serum antibody. The absence of color, or a weaker intensity of color, indicated that BTV-specific antibody was present in the test serum. The results were determined by the percent inhibition (PI) by the test serum of binding of the monoclonal antibody provided in the kit. A test sample was considered to be positive for antibody to BTV if the PI was equal to or greater than 50%. Samples with PI's of less than 50% were negative.

b. Agar gel immunodiffusion (AGID)

Positive samples were confirmed with an AGID test kit purchased from Veterinary Diagnostics, Inc. (Colorado). This kit contains group-specific bluetongue antigen that can detect all BTV serotypes. However, this antigen can also show cross-reaction with EHDV. The test was conducted following manufacturer's directions. Briefly, a seven-well pattern (six outside wells and a center well) was cut into a gel plate made with 0.9% agarose agar in physiological saline (0.85% NaCl in distilled water). The test antigen (25 ul) was placed in the center well, while the reagent serum (25 ul) and test serum (25 ul) were placed in alternating wells around the center well. These reagents

diffused into the agar gel of the wells and formed an insoluble precipitin line which could be seen within a few hours after the wells are filled, reaching an optimum distinct precipitin line at 24 hours at 24°C. A high intensity light source or narrow beam of light was used to examine the precipitin reaction. In a positive reaction, the control lines joined with and formed a continuous line with the precipitin line that had formed between the antigen and test serum. In a negative reaction, the control lines extended into the test sample well without bending or forming a defined "hook" at the tip of the line.

6. Reverse transcription-polymerase chain reaction (RT-PCR)

A few larval pools were simultaneously tested by reverse transcription-polymerase chain reaction by Dr. William Wilson at the USDA/ARS-ABADRL in Laramie, Wyoming. Nine specimens were tested directly from the larval pool and four specimens were tested after three blind passages in BHK-21 cells. The results are included in the summary table of positive pools. The PCR technique utilized two sets of specific primers amplifying regions of the NS1 gene of BTV and the procedure is published elsewhere (Wilson et al., 1992).

7. Determination of minimum field infection rate

When analyzing insect sample pools that may be of variable size, the minimum field infection rate (MFIR) can be calculated which estimates the infection rate in a population of organisms (Walter et al., 1980). The MFIR was obtained by dividing the number of positive pools by the total number of insects processed. The true number of positive members in the pool cannot be calculated without individually testing each member. The MFIR assumes that in a positive pool, a minimum of one of the members of that pool is positive.

8. Statistical analyses

Statistical analyses were performed using SigmaStat Statistical Analysis System, Version 1.01 (Baylor College of Medicine Computer Resource Center). Categorical variables were compared for a significant difference using the Chi Square (X^2) test or the Fisher Exact test. The Fisher Exact test was used when the expected number of observations was less than five in one or more cells of the contingency table. In this case, the Chi Square statistic may not yield an accurate p value. The Z test was used when comparing the proportions of two groups found within a single category, for a significant difference.

Results

1. Sentinel animals

Baseline serum samples were taken on 4/13/92 for the cattle and 6/22/92 for the sheep. Subsequent samples were collected at monthly intervals for eight months (Table 2.2). Calf #508 at the Howard farm seroconverted; antibodies to BTV were detected on 10/23/92 by cELISA and this result was confirmed by AGID. Calf #507, at the Ausman farm, appeared to seroconvert; when tested by cELISA, the test appeared to be positive on 9/4/92 but this was not confirmed by the AGID or subsequent cELISA. Herd animals under two years of age were sampled at two of the sites (Howard and Wagner) 5/19/92 for a baseline reading. Sixteen calves at the Wagner site and 30 calves at the Howard site were bled. All were tested by cELISA, and all were negative. All four herds were then sampled on 11/10/93. Sixteen calves were bled at the Wagner site, 28 from the Howard site, 15 from the Fields site and 10 from the Ausman site. The animals from the Howard and Wagner farms that were tested were the same animals as those tested in the spring. All serum samples were assayed using the cELISA; 6 of the 69 samples were positive (not shown). Four of the positive animals were at the Fields farm, one at the Ausman farm and one at the Howard farm. None of the calves at the Wagner farm seroconverted. All four of the calves at the Fields farm that tested positive were 8 months

of age or younger; presumably they were exposed to BTV during the summer.

2. Larval collections

A total of 62,050 larvae were sampled at two and three breeding sites in 1991 and 1992, respectively. In 1991, the Ausman and Fields sites were tested from February through the end of August. The number of larvae collected from the Ausman site was 7200, while the number of larvae collected from the Fields site was 9700. In 1991, each pool contained approximately 25 larvae, therefore, 16,900 total larvae were tested. In 1992, the Ausman, Fields and Howard sites were sampled from February through the end of August. The number of larvae collected from the Ausman site was 14,300, the number of larvae collected from the Fields site was 22,850, and the number of larvae collected from the Howard site was 8000. In 1992, each pool contained approximately 50 larvae; therefore, 45,150 total larvae were tested.

3. Adult collections

The adult *Culicoides variipennis* collected in the *Culicoides* host preference and temporal feeding activity study (Chapter III) were used to provide evidence of BTV transmission in the Ausman and Fields study areas. The results from this study will be discussed in detail later. The study was conducted in the summer of 1992 for four months at the Fields site and three months at the Ausman

site. Briefly, 995 *C. variipennis* were collected at both sites and sorted into pools ranging in size from 1-50.

4. Diagnostic assays

Eighty-seven larval pools tested BTV-positive by at least one diagnostic test. These eighty-seven pools are shown by date and site, in Appendix A. Thirty-four pools were from 1991 (Table 2.3), while 62 pools were from 1992 (Table 2.4). In 1991, the total number of positive pools at the Fields and Ausman sites were highest in June (10 pools) and August (13 pools). In 1992, the total number of positive pools at the Fields, Ausman, and Howard (Red Barn) sites were highest in May (13 pools) and June (25 pools). The highest number of positive pools were collected from the Ausman site in both August, 1991 (13 pools) and in June, 1992 (13 pools). Twenty-four pools were from the Fields site, 53 pools were from the Ausman site, and 10 were from the Howard (Red Barn) site. Twenty-eight pools were from the water sites, 28 from the waterline sites and 31 from the mud sites.

Sixty-three larval pools tested positive by ELISA; four of these pools were repeat positive by ELISA. One pool, negative by ELISA initially, was positive by PCR and positive on a repeat ELISA. Examples of ELISA values for all pools tested, along with site of collection and date of collection are noted in Appendix B.

RNA hybridization assay yielded 19 positive larval pools; 3 from larval pools directly, 3 from BHK-21 cell passages, 1 from Vero cell passages, 6 from C6/36 cell passages, and 5 from more than one type of cell or larval pool directly. A characteristic RNA hybridization pattern can be seen in Figure 2.11.

Indirect FA assay yielded 2 positive larval pools. The PCR results varied on early specimens due to the assay's experimental status as a diagnostic test for field-collected vector specimens; thus, only 9 larval pools were tested by PCR.

The concordance of positive larval pools with each diagnostic test is shown in Table 2.5. Eleven larval pools tested positive by two tests. Five larval pools tested positive by two RNA hybridization assays. Three pools tested positive by the PCR assay and ELISA, while three pools tested positive by the ELISA twice. One larval pool was positive for three tests including the PCR test and two ELISA tests.

Borderline positives (i.e. sample mean ELISA optical density [o.d.] value = background mean ELISA o.d. value plus 3 standard deviations [SD]) were not included as positive larval pools. These were designated as equivocal values and the assays were repeated with subsequent negative results.

Sixty-three adult *C. variipennis* pools of 266 total pools (23.6%) tested positive for BTV by ELISA at the Fields farm site (Table 2.6). Twenty-eight of these pools were collected from the calf, while two pools were collected from the sheep. At the Ausman farm site, twenty-one pools were collected from the calf and twelve pools were collected from the sheep for a total of thirty-three positive pools.

5. Minimum field infection rates

The minimum field infection rates (MFIRs) in the larval pools at the farm sites are shown for 1991 in Table 2.3 and for 1992 in Table 2.4. In 1991, at the Ausman site, the MFIRs varied from 0.4% in February, 0.0% in March and April, 0.8% in May, 0.48% in June, 0.0% in July, and 0.33% in August. The MFIR for the year at this site was 0.24%. At the Fields site, in 1991, the MFIRs varied from 0.0% in February and March, 0.44% in April, 0.31% in May 0.10% in June, and 0.0% in July and August. The MFIR for the year at this site was 0.12%.

In 1992, at the Ausman site, the MFIRs varied from 0.15% in February, 0.5% in March, 0.0% in April, 0.07% in May, 0.15% in June, 0.09% in July, and 0.26% in August. The MFIR for the year at this site was 0.22%. At the Fields site, in 1992, the MFIRs varied from 0.33% in February, 0.09% in March 0.31% in April, 0.14% in May, 0.32% in June, 0.06% in July, and 0.0% in August. The MFIR for the year at

this site was 0.09%. In 1992, at the Howard farm, no larvae were collected in February and March. The MFIR in April was 0.0%, 0.15% in May, 0.14% in June, 0.20% in July, and 0.14% in August. The MFIR for the year at this site was 0.13%.

MFIR's for the collected adult *C. variipennis* pools (Chapter III) at both farm sites are shown in Table 2.6. At the Fields site, from the calf, the MFIR in June was 1.32%, 4.59% in July, 5.83% in August, 4.91% in September and the total MFIR was 4.55%. At the Fields site, from the sheep, the MFIR in June was 0% in June and September, 2.85% in July, and 4.54% in August. At the Ausman site, from the calf, the MFIR in June was 0%, 8.0% in July, and 9.2% in August. At the Ausman site, from the sheep, the MFIR in June was 0%, 27.7% in July, and 18.18% in August.

MFIR's can also be used to compare the diagnostic assays. Utilizing the ELISA test as the only BTV detection test, the MFIR would be 0.10%. Utilizing RNA hybridization as the only BTV detection test, the MFIR would be 0.03%; utilizing virus isolation techniques only, the MFIR would be 0.003%.

6. Statistical analyses

The monthly MFIR's in larval pools collected from the Ausman and Fields sites were compared in 1991 (Table 2.3). Chi Square (X^2) analysis revealed that the MFIR's in the two areas only differed in the month of June ($p=0.018$). In

June, the MFIR for the Ausman site (0.80) was nearly three times as high as the MFIR for the Fields site (0.31). The total MFIR's for 1991 for the Ausman and Fields sites differed ($\chi^2 = 5.903$; $p = 0.015$). The MFIR at the Ausman site was 0.24 and the MFIR at the Fields site was 0.12 for the year. The z test analysis also calculated that these rates differed significantly for the month of June ($z=2.452$; $p=0.014$) and for the year ($z=2.809$; $p=0.005$).

In 1992 (Table 2.4), Chi Square analysis revealed that the yearly MFIR's at the three farm sites differed ($\chi^2 = 10.079$; $p = 0.006$). The MFIR's of the sites were then compared. The MFIR's of the Ausman and Fields farm differed significantly ($z=2.691$; $p=0.007$). However, the MFIR's of the Fields and Howard farm ($z=0.40$; $p=0.968$) and the Ausman and Howard farm ($z=1.597$; $p=0.110$) did not differ statistically.

D. Discussion

BTV remains endemic in many areas of the world, including Colorado, even though the vectors have specific periods of inactivity each winter due to adverse environmental conditions. To determine the mechanism of BTV transeasonality in Colorado, an extensive prospective study was conducted in BTV endemic areas. Standard virus isolation and newer molecular methods were used to determine the overwintering mechanism of BTV.

Field larvae were collected weekly from three farm sites and tested. Approximately 16,900 larvae were tested in 1991 while approximately 45,150 larvae were tested in 1992 for a total of 62,050 larvae over a two year period. Eighty-seven larval pools tested BTV-positive by at least one diagnostic assay test; 11 pools tested positive by two assay tests. One larval pool was positive for three tests including the PCR test and two ELISA tests. Borderline positive results occurred in all of the assays tested; typically, on repeat assay these samples yielded negative results.

The ELISA detected sixty-three BTV-positive pools, RNA hybridization detected 19 positive pools, IFA detected two positive pools and PCR detected 7 positive pools. Pools testing positive by ELISA and FA reflect the fact that the viral antigens are intact; pools testing positive by RNA hybridization and PCR reflect the fact that viral RNA is present in some form. This detection rate follows supportive data that shows the ELISA, which detects the more abundant viral antigen, should be more sensitive than RNA hybridization which detects the nucleic acid. Virus isolation should be the least sensitive in this case because infectious virus needs to be present.

The diagnostic test concordance of positive larval pools was low. Five larval pools tested positive by two RNA

hybridization assays. Two pools tested positive by both PCR assay and ELISA, while three pools tested positive twice by ELISA. To explain the lack of concordance, low-levels or noninfectious BTV may be present in the larvae that, when stimulated by morphogenesis or change in environmental temperature, are activated to produce infectious virus. Other reasons include the possibility of cross-reactions of certain tests, such as the ELISA, with a related orbivirus previously uncharacterized. The tests themselves have known specificities and sensitivities when using laboratory strains of BTV, but these standards may differ when testing field specimens. None of the newer tests such as the ELISA and RNA hybridization have been tested under field conditions.

The BTV prevalence rate differed significantly by farm site but not by collection site on each farm site. Sixty-one percent of the positive pools were collected from the Ausman farm, 27% from the Fields farm, and 12% from the Howard (Red Barn) site ($p < 0.001$). Almost equal percentages of pools were collected from the mud (32%), waterline (32%), and water (36%) ($p > 0.05$).

Minimum field infection rates at the sites in 1991 at the Ausman site ranged from 0.0% to 0.8% for an average of 0.24%, and the Fields site from 0.0% to 0.44% for an average of 0.12%. In 1992, MFIR's at the Ausman site varied from

0.0% to 0.5% for an average of 0.22%; MFIR's at the Fields farm varied from 0.0% to 0.32% for an average of 0.09%; and the Howard farm varied from 0.0% to 0.2% for an average of 0.13%. During both 1991 and 1992 MFIR's were significantly higher at the Ausman farm than at the other farm sites.

Chi Square analysis showed a significant difference between the MFIR's in 1991 at the Ausman and Fields site in the month of June ($p = 0.018$) and for the year ($p=0.015$). There was also a significant difference between the MFIR's at the Ausman and Fields farm for the year ($p=0.007$). In 1992, Chi Square analysis showed a significant difference between the MFIR's at the three farm sites for the year ($p = 0.006$).

BTV was being transmitted in the study areas; this was demonstrated by both seroconversion in six of the herd animals as well as seroconversion in at least one of the sentinel calves. In addition, 23.6% of adult *C. variipennis* pools collected weekly from two of the farm sites were found to be positive for BTV when tested by antigen-capture ELISA (see Chapter III for father results).

To summarize the experiments, a time course analysis of the transeasonality events for 1992 can be seen in Figure 2.12. This depicts the time periods for the various collections and seroconversions of the sentinel and herd calves after September. This timeline can be combined with

Figure 2.13 which compares the minimum number of larvae positive at 3 sites in 1992 (A) with the number of adult *C. variipennis* positive at 2 sites (B) in 1992. Utilizing these two figures, one can conclude that BTV prevalence in the larvae peaked in June, while BTV prevalence in the adult *C. variipennis* peaked in July. Sentinel calf conversion occurred in September and October and it can be assumed that BTV infection in these calves occurred approximately in August.

Therefore, the timing of the results seem to fit the hypothesis that BTV overwinters in the larvae. A proposed mechanism would involve small numbers of vertically-infected larvae (many larvae would die off during the winter, resulting in a very low number of larvae actually infected with BTV), that emerge as infected adults in early spring. After 2 to 3 breeding cycles, BTV could infect more *Culicoides* larvae through vertical transmission (a viral amplification of sorts even at a low level). This would explain why the BTV-positive larval peak is seen in June. Then this group of larvae become adults. These adults could then infect the vertebrates with BTV in August resulting in seroconversion of the animals in September and October. BTV-infected *Culicoides* adults would then vertically transmit BTV again resulting in BTV-infected larvae, that overwinter, thereby starting the cycle over again.

This proposed cycle of events would explain why previous studies have not been able to show vertical transmission in the field; most studies are instituted during or at the end of BTV epidemics. These often occur in September and October, which would be after infected larvae are at their peak. In addition, the prevalence of BTV infection in larvae is so low that thousands would need to be tested to find BTV. For example, in one study in Australia, 170,000 *Culicoides* adults were sorted to species and tested, resulting in a single isolation of BTV (Standfast et al., 1992). The investigators concluded that the collection of insects in the field and their processing for virus isolation is not an efficient use of resources.

However, these preliminary positive results could be used to focus the investigation. In both 1991 and 1992 the Ausman farm had significantly higher MFIR's than the other farm sites. More extensive studies could be concentrated at this area in the early months of May through August. To prove the overwintering hypothesis, infectious virus must be isolated from larvae or adult flies reared in the laboratory from field-caught larvae. In the future, larvae could be collected and reared to the adult stage, identified to species, and then screened with the ELISA. Portions of positive pools could be immediately injected into embryonated chicken eggs, the gold standard for virus

isolation. This method was not used in this investigation due to the enormous cost and time commitment the assay would have placed on the study. Unfortunately, larval pools in the study were placed at -20°C after processing, resulting in a low chance of recovering infectious virus using the ECE system at a later date. Molecular tests such as RNA blot hybridization and the RTC-RT-PCR assay (described in Chapter V) may be the best tests to further investigate mechanisms of transeasonality, if a transcriptionally latent viral state is involved.

COLORADO

Field Sites

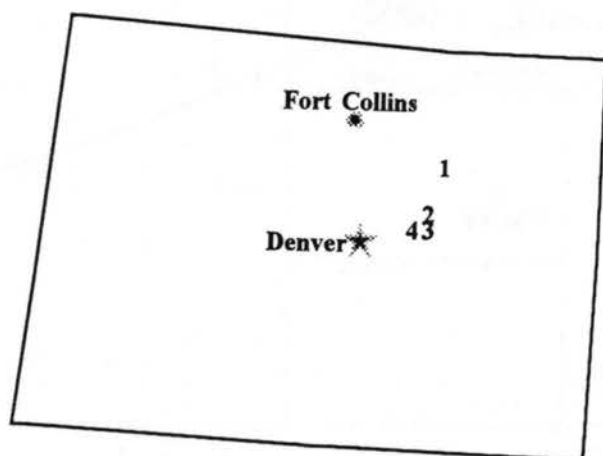


Figure 2.1. Field study sites in Colorado. The study sites are represented by number: Study site 1--Fields farm, study site 2--Howard farm, study site 3--Wagner farm, and study site 4--Ausman farm.

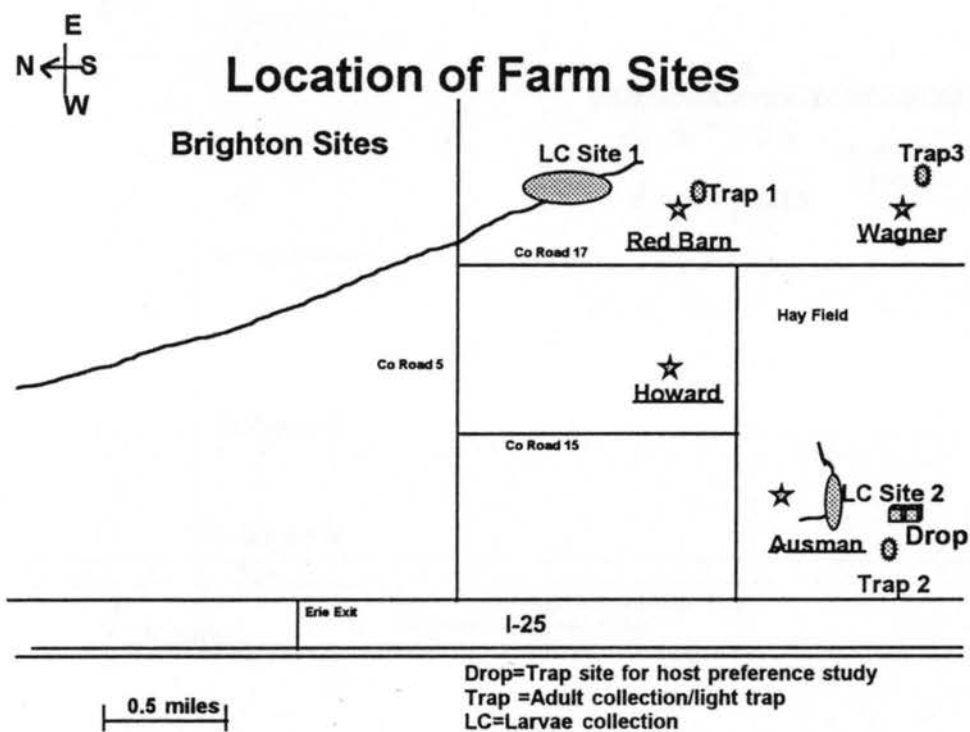


Figure 2.2. Detailed map of Ausman, Wagner, and Howard farm sites

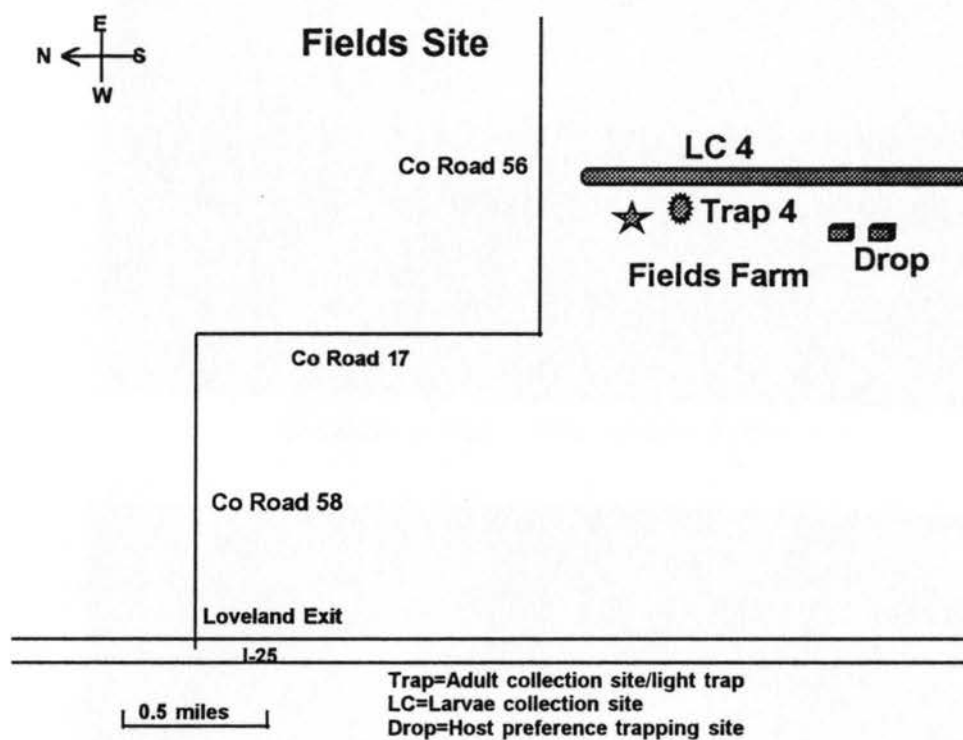


Figure 2.3. Map of Fields farm site



Figure 2.4A. The Ausman farm.



Figure 2.4B. The Howard farm



Figure 2.4C. The Wagner farm.



Figure 2.4D. The Fields farm.



Figure 2.5. Characteristic area of pasture with poor drainage found at all four farm sites.



Figure 2.6A-E. Five sampling sites at the Fields farm *Culicoides* breeding site.



Figure 2.7. Three sampling areas tested at each sampling site: 1) waterline, 2) water, and 3) mud

Table 2.1. Key to pool letters by farm and study site

Fields		Ausman		Howard	
Pool Letter	Site	Pool Letter	Site	Pool Letter	Site
A	W 1	G	W 1	RA	Water 1
B	W 2	H	W 2	RB	Water 2
C	WL 1	I	WL 1	RC	WL 1
D	WL 2	J	WL 2	RD	WL 2
E	M 1	K	M 1	RE	M 1
F	M 2	L	M 2	RF	M 2
M	W 3	T	W 3	RG	W 3
N	W 4	U	W 4	RH	W 4
O	WL 3	V	WL 3	RI	WL 3
P	WL 4	W	WL 4	RJ	WL 4
Q	M 3	X	M 3	RK	M 3
R	M 4	Y	M 4	RL	M 4
AA	W 5	DD	W 5	RM	W 5
BB	WL 5	EE	WL 5	RN	WL 5
CC	M 5	FF	M 5	RO	M 5
Z	W 6	S	W 6	RP	W 6

W = Water site
 WL = Waterline site
 M = Mud site

Sites 1-6 represent different sampling areas of the breeding sites at the study farms.



Figure 2.8. CDC light trap at a breeding site.

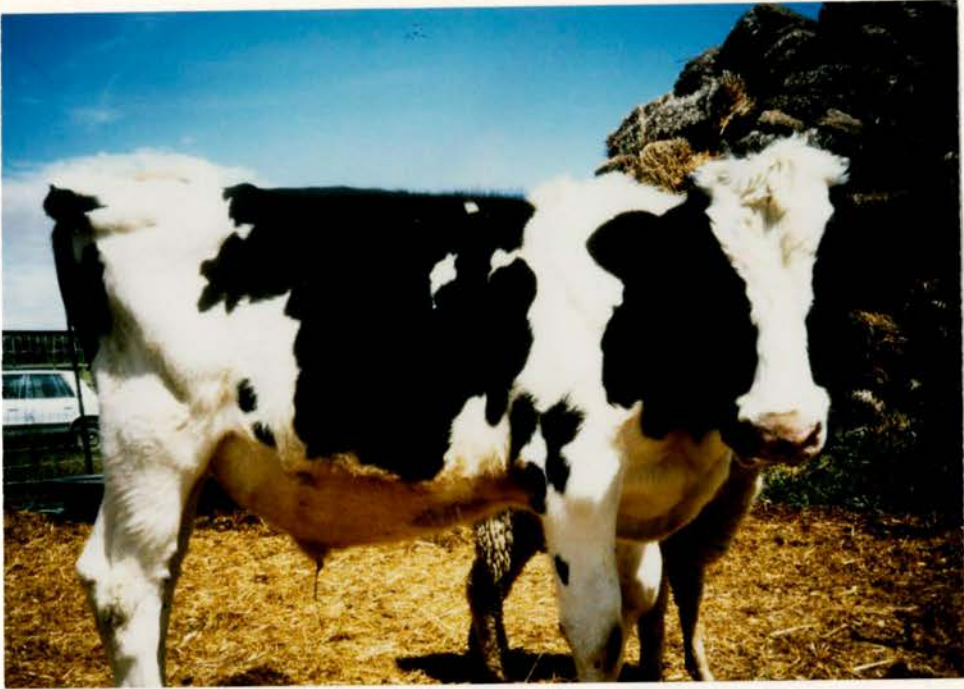
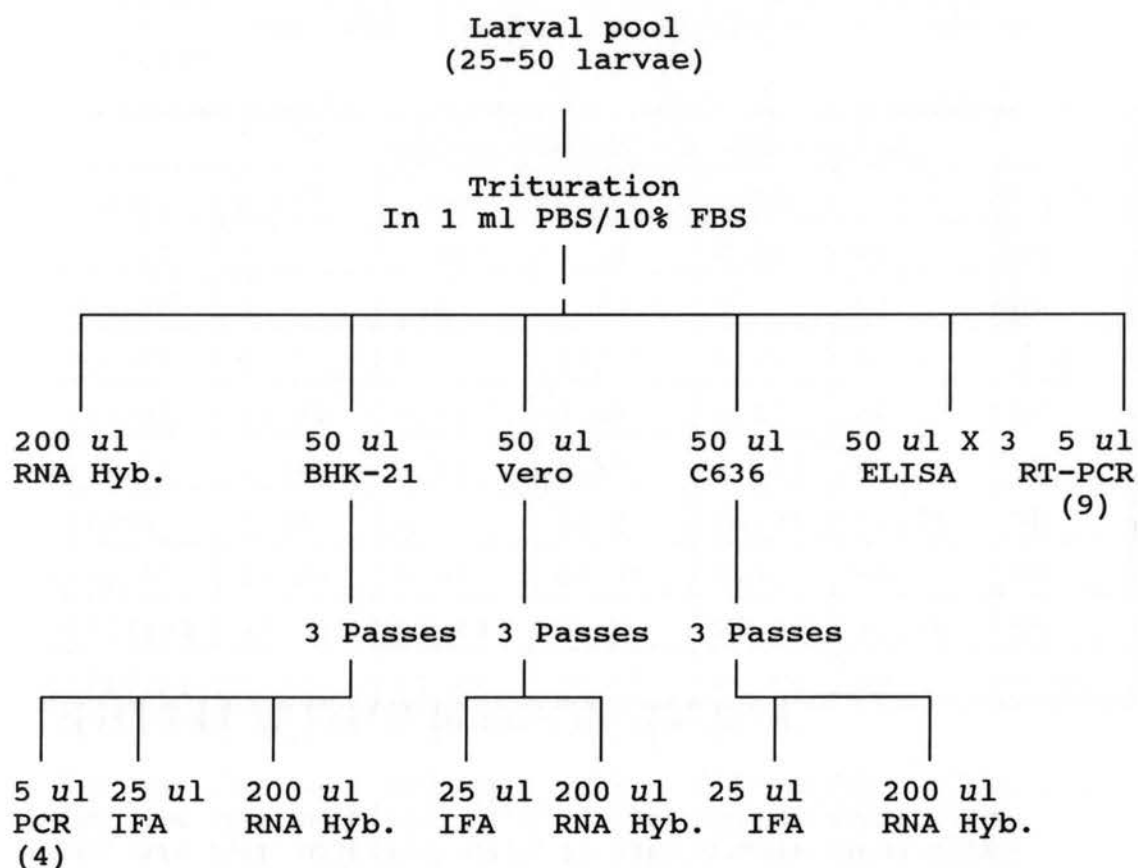


Figure 2.9A. Picture of sentinel calf



Figure 2.9B. Picture of sentinel sheep.



RNA Hyb. = RNA hybridization assay
 ELISA = Antigen capture ELISA
 IFA = Indirect fluorescent antibody test
 BHK-21 = Virus isolation in BHK-21 cells
 Vero = Virus isolation in Vero cells
 C636 = Virus isolation in C636 cells
 RT-PCR = Reverse transcription polymerase chain reaction assay on only 9 specimens directly from the larval pool and 4 specimens from the BHK-21 virus isolation assay

Figure 2.10. Flow chart of the vector diagnostic assays used in the study.

Table 2.2. Direct EIA results on sentinel animal serum samples. The EIA result is measured in percent of inhibition.

Animal tested (% Inhibition)						
Date	C 497	C 499	C 507	C508	S 1	S 2
4/13/92	0	0.86	1.35	4.09	ND	ND
5/19/92	0	1.4	2.48	0	ND	ND
6/15/92	0	0	13.67	5.47	14.0	0.0
7/23/92	11.15	3.09	5.97	6.67	ND	ND
8/19/92	5.04	2.95	1.87	21.01	ND	ND
9/4/92	1.38	0	76.53	14.76	11.65	ND
9/25/92	13.09	5.94	42.56	9.23	ND	ND
10/23/92	32.18	11.42	12.8	94.2*	32.18	ND
11/10/92	9.17	11.19	15.51	68.69	ND	5.94

* Confirmed by the BTID test as positive

C 497 was the sentinel calf at the Fields farm site
 C 499 was the sentinel calf at the Wagner farm site
 C 507 was the sentinel calf at the Ausman farm site
 C 508 was the sentinel calf at the Howard farm site
 S 1 was the sentinel sheep at the Fields farm site
 S 2 was the sentinel sheep at the Ausman farm site

ND = Not Done

Table 2.3: Minimum field infection rates (MFIRs) in larval pools tested at 2 breeding sites during 1991.

		Feb.	March	April	May	June	July	August	Total
Aus	#+/#t	2/500	0/0	0/0	2/250	5/1025	0/1800	13/3875	22/7200
	MFIR	0.40	0.00	0.00	0.80	0.48	0.00	0.33	0.24
Fie	#+/#t	0/325	0/0	4/900	3/950	5/5050	0/1550	0/675	12/9700
	MFIR	0.00	0.00	0.44	0.31	0.10	0.0	0.0	0.12
Total	#+/#t	2/825	0/0	4/900	5/1200	10/6075	0/3350	13/4550	34/16900
	MFIR	0.24	0.0	0.44	0.42	0.16	0.0	0.28	0.2
	X ²	0.172	ND	ND	0.251	5.614	ND	1.239	5.903
	p	0.678	ND	ND	0.617	0.018**	ND	0.266	0.015**

MFIR is expressed as:
$$\frac{\text{Number of positive pools (\#+)}}{\text{Total number of larvae collected (\#t)}} \times 100 = \%$$

X² values are calculated with 1 degree of freedom

ND = Not Done/Not able to calculate

** = p value ≤ 0.05 (Significant)

Aus = Ausman farm site

Fie = Fields farm site

Table 2.4: Minimum field infection rates (MFIRs) in larval pools tested at 3 breeding sites during 1992.

		Feb.	March	April	May	June	July	August	Total
Au	#+/#t	2/1300	5/1000	0/500	5/6750	13/8900	3/3250	3/1150	31/14300
	MFIR	0.15	0.50	0.00	0.07	0.15	0.09	0.26	0.22
Fi	#+/#t	1/300	1/1150	4/1300	2/1450	10/3050	3/4900	0/2150	21/22850
	MFIR	0.33	0.09	0.31	0.14	0.32	0.06	0.00	0.09
Ho	#+/#t	ND	ND	0/1400	6/3950	2/1450	1/500	1/700	10/8000
	MFIR	ND	ND	0.00	0.15	0.14	0.20	0.14	0.13
To	#+/#t	3/1600	6/2150	4/3200	13/12150	25/13400	7/8650	4/4000	62/45150
	MFIR	0.19	0.28	0.13	0.11	0.19	0.08	0.04	0.14
	X ²	0.009	1.948	5.836	1.556	4.218	1.161	5.247	10.079
	p	0.925	0.163	0.054	0.459	0.121	0.560	0.073	0.006**

MFIR is expressed as:
$$\frac{\text{Number of positive pools (\#+)}}{\text{Total number of larvae collected (\# tot)}} \times 100 = \%$$

ND = Not done

X² value calculated with two degrees of freedom

** = p value $\leq$ 0.05 (Significant)

Au = Ausman farm site

Fi = Fields farm site

Ho = Howard farm site

To = Total



Figure 2.11. Characteristic slot blot hybridization of RNA extracted from larval pools utilizing a ^{32}P labeled probe. Slots A1-A13, B1-B13, and C1-C13 are various larval pools RNA extracted, blotted and hybridized during the study. Specific pools are as follows: Slot B1--pool 11468 (extraction from larval-pool-infected BHK-21 cells), Slot C11--pool 11404 (direct extraction from larval pool), Slot A14--positive control RNA extracted from BTV-17 infected BHK-21 cells, Slot B14--negative control RNA extracted from uninfected BHK-21 cells, Slot C14--negative control RNA extracted from an uninfected pool of larvae.

Table 2.5. Concordance of positive larval pools. Numbers of positive pools are listed along with their concordance.

	ELISA	RNA	IFA	PCR
ELISA	63	1	0	3
RNA	1	19	0	0
IFA	0	0	2	0
PCR	3	0	0	7

ELISA = Enzyme-linked immunosorbent assay

RNA = RNA blot hybridization

IFA = Indirect fluorescent antibody test

PCR = Polymerase chain reaction

Table 2.6. Minimum field infection rates (MFIR's) in adult pools collected from 2 farm sites during 1992. This table is discussed in more detail in Chapter II.

		June	July	August	September	Total
Fields	Calf	1/76	9/196	7/120	11/224	28/616
	MFIR	1.32	4.59	5.83	4.91	4.55
	Sheep	0/3	1/35	1/22	0/18	2/78
	MFIR	0.00	2.85	4.54	0.00	2.56
	p value	>0.999	0.996	0.783	0.735	0.628
Ausman	Calf	0/2	14/175	7/76	ND	21/253
	MFIR	0.00	8.00	9.20	ND	8.30
	Sheep	0/1	10/36	2/11	ND	12/48
	MFIR	0.00	27.70	18.18	ND	25.00
	p value	ND	0.009**	0.351	ND	0.008**

MFIR = Minimum field infection rate

MFIR is expressed as:
$$\frac{\text{Number of positive pools (\#+)}}{\text{Total number of adults collected (\#t)}} \times 100 = \%$$

X² values are calculated with 1 degree of freedom

ND = Not Done/ Not able to calculate

** = p value $\leq$ 0.05 (Significant)

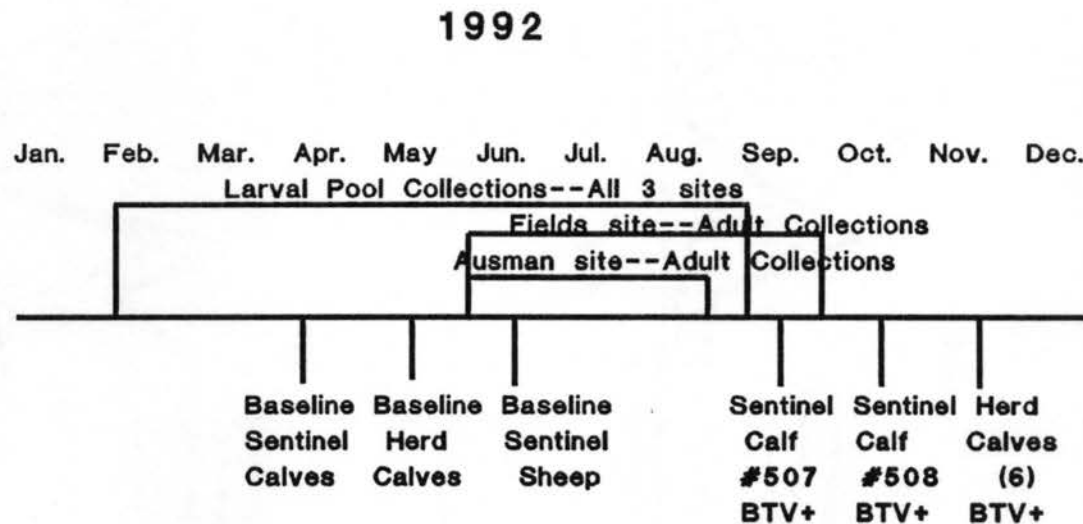
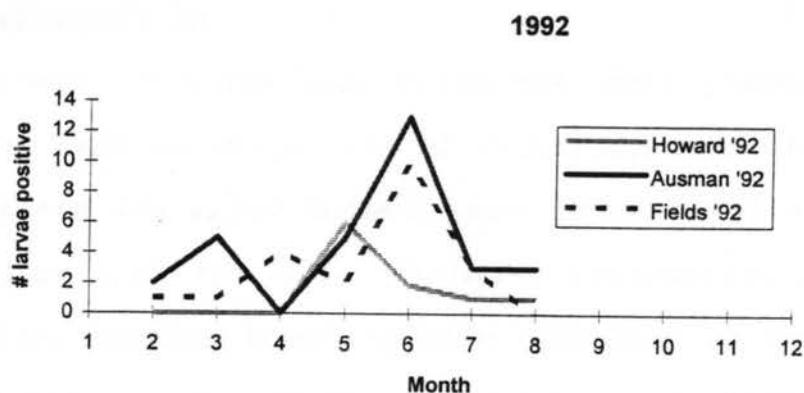


Figure 2.12. Time course analysis of transeasonality events for 1992

A.



B.

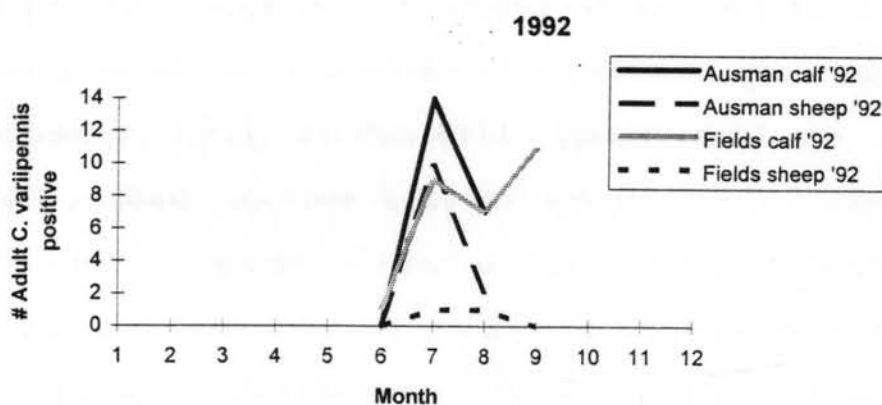


Figure 2.13. Comparison of the minimum number of larvae positive (MFIR) at 3 sites in 1992 (A) with the number of adult *C. variipennis* positive at 2 sites (B) in 1992.

III. *CULICOIDES* HOST PREFERENCE AND TEMPORAL FEEDING ACTIVITY STUDY

A. Introduction

Little is known concerning the host preferences and temporal feeding activities of *C. variipennis* in Colorado. Since these are major determinants of vectoral capacity of *C. variipennis* for BTV, intensive prospective studies of *Culicoides* feeding behavior were conducted on two premises in Colorado. The study was similar in design to one conducted by Schmidtman et al. (1980b) in New York, in which caged animals and drop traps were used to demonstrate that *C. venustus* fed on pastured calves. The drop trap method permits unobstructed access of insects to selected bait animals, and thus biologically-relevant data regarding host-exposure rates to temporal insect activity can be gathered. Such studies provide unequivocal evidence of blood feeding by putative vectors on vertebrate hosts. In Australia, the drop trap method proved to be quite effective for collection of engorged mosquitos but not for midges because of the low rates of attack encountered (Muller and

Murray, 1977). The drop trap method has more recently been used to collect blood-feeding flies on bait animals in an area of active BTV and EHDV transmission (Mullens and Dada, 1992a).

Culicoides species are opportunistic feeders that differ in host-specificity, diel activity (day-time or night-time), site on the host at which they feed, etc., and differences may also be observed between individuals of the same species (Kettle, 1977). Host size, but not host color, has been found to be a significant factor in attracting *Culicoides* (Humphreys and Turner, 1973). Sheep have been found not to be as attractive to *Culicoides* as cattle, thus when sheep and cattle are in close proximity to each other, sheep may be protected because the vectors will be preferentially attracted to the cattle (DuToit, 1962). Some *Culicoides* species tend to feed near ground level, while others feed at higher altitudes (Tanner and Turner, 1974). *Culicoides* may be attracted to specific areas of the host anatomy, such as the underside of the abdomen in sheep, but may not be attracted to non-wooled areas such as the head or legs (Schmidtman et al., 1980a). In terms of diel activity, the greatest number of airborne *C. variipennis* are observed from one hour before to one hour after sunset in

May, September and October, and directly following sunset in June, July and August (Barnard and Jones, 1980a).

Several predominant species of *Culicoides* can be collected in the western U.S. In light trap collections from southeastern Nebraska, *C. crepuscularis*, *C. stellifer*, *C. haematopodus*, *C. variipennis* and *C. obsoletus* were the predominant species (Pappas and Pappas, 1989). In suction trap collections from southern California over a two year period, *C. (Selfia) brookmani*, *C. variipennis*, *C. (Avaritia)* sp. and *C. lahontan* were the predominant species (Mullens and Dada, 1992b). In one study in Colorado, over 120,000 insects were collected including *Culicoides* species; 59% of the total insects were *C. (Selfia)* spp. complex (Kramer et al., 1990). *C. (Selfia)* spp. harbored for 89.6% of the arboviruses isolated from these collections. Other insects collected included *C. variipennis*, *C. stellifer*, and *C. cockerellii*.

To study *Culicoides* host preference and temporal feeding activity in Colorado, intensive prospective studies were conducted with USDA/ARS-Arthropod-borne Animal Disease Research Laboratory (ABADRL) scientists on two farm premises in Colorado. The research hypotheses for this study were 1) a greater number of *Culicoides variipennis* would be attracted to the calf than the sheep at both farm sites, 2) larger numbers of *C. variipennis* should be found in late

summer at both farm sites as well as during the sunset time period, and 3) there should be a low BTV prevalence rate in collected *C. variipennis* due to the previous documented presence of circulating BTV in the two areas. To study these hypotheses insects attracted to sentinel animals were sampled at 2 farm sites to characterize and compare host preferences, seasonal activity periods, and site differences. Insects were collected, identified, and prevalence of BTV determined by a capture-antigen ELISA.

B. Materials and Methods

1. Sites

Two of the same sites used in the transeasonality study described in chapter II were used for this study; the Ausman farm near Brighton, Colorado, and the Fields farm near Loveland, Colorado.

2. Vertebrates used in traps

One Holstein calf and one Dorset ewe were kept at each farm. These were the same animals that acted as sentinel animals in the transeasonality study. The calves were equal in size and increased from approximately 100 kg to 200 kg over the course of the study. The calf at the Fields farm was almost all black, while that at the Ausman farm was about 50% black. The ewes were off-white, equal in size, and ranged from 30 kg to 80 kg during the course of the

study. The ewes were not shorn except for a 5" X 5" spot on the neck used for drawing blood.

3. Traps

Two drop traps were located at each of the premises (Figures 3.1A and 3.1B). Each drop trap consisted of 2 permanent wooden crossbars with pulleys and ropes that suspended a frame-work of aluminum poles, permitting the frame-work to be raised and lowered. Fine-gauge (<60 mesh) mosquito nets were sewn together to form a tent-like fabric, which was secured to the frame-work forming an insect-proof barrier around the animals. A zipper along the height of the net on one side gave access to the interior of the trap.

4. Sampling schedule

The areas were sampled twice weekly, typically on Monday and Wednesday nights. Trapping started on June 29, 1992, and continued until August 19, 1992, at the Ausman site, and September 22, 1992, at the Fields site. If bad weather interfered with more than 2 sampling periods at a site on a certain date, sampling for that site on that date was cancelled and noted as not done in the results. Sampling periods were 30 minutes in length and started at thirty minutes prior to local sunset. Five periods were completed per night.

5. Trapping protocols

Animals were placed in their respective stanchions, brushed down to dislodge insects already present (Figure 3.2A), and exposed to the open air for 10 minutes (Figure 3.2B). Personnel vacated the immediate area for these ten minutes to preclude affecting the study. After animal exposure, the cages were lowered for ten minutes to allow the insects to complete engorgement (Figure 3.2C). All insects were collected from the walls of the cages with a customized vacuum aspirator (Schmidtman et al., 1980) (Figure 3.2D). Vials containing the insects were identified by the farm site, time period, and type of animal from which they were collected. The cages were then raised prior to the beginning of the next period and the cycle was repeated. Head lamps were used to collect the insects after dark; the lamps were turned off at all other times. The vials containing live insects were transported to USDA/ARS-ABADRL, Wyoming. Insects were identified on ice to the genus of mosquitoes, *C. variipennis*, *Culicoides* species other than *C. variipennis*, and other insects, formed into pools, and stored frozen at -70°C .

6. ELISA

The insect pools were screened using the antigen-capture ELISA to determine prevalence of BTV in hematophagous arthropods at each farm site. The procedure

was as described in detail in chapter II. Briefly, after trituration of the sample pool in phosphate buffered saline using a mortar and pestle, the sample pool was sonicated for 30 sec on ice. Fifty ul of the sample was then aliquoted into each well which had previously been coated with anti-BTV IgG antibody, incubated overnight at room temperature and washed 5 times in wash (PBS-Tween-20) solution. After incubation of the sample for one hour at 37°C, the plate was washed five times with wash solution. Anti-BTV-10 mouse ascitic fluid was added to each well (50 ul each), incubated and washed 5 times with wash solution. Goat anti-mouse antibody conjugated to horseradish peroxidase was added to each well (50 ul each), incubated and washed 5 times. Substrate TMB (3,3',5,5'-tetramethylbenzidine) was added and incubated for 40 min. Absorbance was measured at a wavelength of 450 nm in a microplate reader. Results were considered to be positive if the absorbance reading was greater than the mean of the negative absorbance reading plus three standard deviations.

7. Minimum field infection rate (MFIR)

When variably-sized sample pools are analyzed, the MFIR can be calculated to estimate the infection rate in a population of organisms (Walter et al., 1980). The MFIR is obtained by dividing the total number of virus positive pools by the total number of organisms processed. The MFIR

was described in Chapter II, and is based on the assumption that at least one infected fly is in a positive pool. More flies could be infected in that same pool, but the exact number cannot be determined.

8. Statistical analyses

Statistical analyses were performed using the SigmaStat Analysis System, Version 1.01 (Baylor College of Medicine Computer Resource Center). Categorical variables were compared for a significant difference using the χ^2 or the Fisher Exact test. The Fisher Exact test was used when the expected number of observations was less than 5 in one or more cells of the contingency table. In this case, the Chi Square statistic may not yield an accurate p value. The t test was used to test the hypothesis that the means of two groups of observations in a data set were equal.

C. Results

1. *C. variipennis*--Fields site

During the study period, 694 *C. variipennis* were collected from the Fields farm site. The numbers of *C. variipennis* collected from the calf varied substantially depending on the period, while the numbers of *C. variipennis* collected from the sheep stayed relatively constant for each period. The total numbers of *C. variipennis* collected from the calf and the sheep are shown in Figure 3.3. From the calf (Table 3.1), 198 flies were collected in period 1,

which decreased to 43 flies in period 5. From the sheep (Table 3.2), 13 flies were collected in period 1, which increased to 20 flies in period 2, decreased to 10 in period 3, and then increased to 16 and 19 flies in periods 4 and 5 respectively.

The nightly period average of *C. variipennis* trapped (total number of flies trapped during the study/number of nights trapping occurred) for each period from the Fields site is shown in Figure 3.4. The nightly period averages from the calf were: 9.9 in period 1, 7.1 in period 2, 6.7 in period 3, 4.9 in period 4 and 2.15 in period 5. Therefore, on an average trap night, the largest number of flies were collected each night from the calf during period 1 and the smallest number of flies were collected during period 5. The nightly period averages from the sheep were: 0.65 in period 1, 1.0 in period 2, 0.5 in period 3, 0.8 in period 4, and 0.95 in period 5. On an average trap night, the largest number of flies were collected each night from the sheep during period 2 and the smallest number of flies were collected during period 3.

There were 20 trapping nights in the study period at the Fields site. Collections were not done on 7/29/92 due to inclement weather. The total number of *C. variipennis* per trap date at the Fields site varied for the calf and the sheep (Figure 3.5). The largest number of flies (136) were

collected from the calf on 9/10/92. In contrast, no flies were collected on 8/10/92. The number of flies collected from the sheep varied from 0 (7/13/92 and 9/17/92) to 14 (7/6/92). The mean number of flies collected per trap night during the study was 30.8 (616/20) from the calf and 3.9 (78/20) from the sheep. Thus significantly more flies were collected from the calf than from the sheep ($X^2 = 49.12$; $p < 0.001$).

2. *C. variipennis*--Ausman site

During the study period, 301 *C. variipennis* were collected from the Ausman farm site. The numbers of *C. variipennis* collected from the calf varied substantially, while the numbers of *C. variipennis* collected from the sheep stayed relatively constant for each period. The total numbers of *C. variipennis* collected from the calf and the sheep are depicted in Figure 3.6. From the calf (Table 3.3), 40 flies were collected in period 1, which decreased to 35 flies in period 2, increased to 74 flies in period 4, and then decreased to 53 in period 5. From the sheep (Table 3.4), 9 flies were collected in period 1, which increased to 14 flies in period 4 and decreased to 8 flies in period 5.

The nightly period average of *C. variipennis* trapped for each period from the Ausman site is shown in Figure 3.7. The nightly period averages from the calf were: 2.5 in period 1, 2.2 in period 2, 3.2 in period 3, 4.6 in period 4

and 3.3 in period 5. In an average trap night, the largest number of flies were collected each night from the calf during period 4 and the smallest number of flies were collected during period 5. The nightly period averages from the sheep were: 0.56 in period 1, 0.5 in period 2, 0.56 in period 3, 0.87 in period 4, and 0.5 in period 5. On an average trap night, the most flies were collected from the sheep during period 4, and the smallest number of flies during period 2.

There were 16 trapping nights in the study period at the Ausman site. The total number of *C. variipennis* per trap date at the Ausman site varied for the calf and the sheep (Figure 3.8). The largest number of flies (95) were collected from the calf on 7/6/92. In contrast, no flies were collected on 7/8/92. The number of flies collected from the sheep varied from 0 (4 trap dates) to 15 (7/6/92). The mean number of flies collected per trap night during the study was 15.8 (253/16) from the calf and 3 (48/16) from the sheep. Thus significantly more flies were collected from the calf than from the sheep ($X^2 = 21.60$; $p < .001$).

Statistical analysis (t test) of the mean *C. variipennis* captures from the calf (Table 3.5) or the sheep (Table 3.6) showed no significant differences between the Fields and Ausman farm sites for periods 1 to 5 during the study. However, statistical analysis (t test) of the mean

C. variipennis captures from the Ausman farm showed significant differences between the calf and the sheep in periods 2 ($p=0.0005$), 3 ($p=0.0021$), 4 ($p=0.0024$), and the total ($p=0.0002$) (Table 3.7). Statistical analysis (t test) of the mean *C. variipennis* captures from the Fields farm showed significant differences between the calf and the sheep for periods 1 ($p=0.04$), 3 ($p=0.04$), and the total ($p=0.04$) (Table 3.8).

3. Other *Culicoides* species

Other species of *Culicoides* caught in the drop-traps included *C. crepuscularis*, *C. haematopodus*, and *C. cockerellii*. The second most abundant *Culicoides* species trapped was *C. crepuscularis*; 283 flies were collected during the study. At the Fields farm, 80 flies were collected from the calf; 120 flies were collected from the sheep. At the Ausman farm, 34 flies were collected from the calf; 49 flies were collected from the sheep. Very few *C. cockerellii* and *C. haematopodus* were collected. Nine *C. cockerellii* were caught from the Fields site only, while 24 *C. haematopodus* were caught from the Fields site and 32 *C. haematopodus* were caught from the Ausman site.

4. BTV prevalence in *C. variipennis*

BTV prevalence in *C. variipennis* at the 2 study sites was measured by the ELISA. ELISA absorbance values for each pool tested in the study with corresponding identification

and pool size are listed in Appendix C. Minimum field infection rates (MFIR) of *C. variipennis* at the two farm sites were calculated (See Figure 2.6 in Chapter II). At the Fields site, the MFIR for *C. variipennis* was 4.32% (30/694). At the Ausman site, the MFIR for *C. variipennis* was 10.5% (33/314). Thus the prevalence rate of BTV in *Culicoides variipennis* was significantly greater at the Ausman site than at the Fields site ($X^2 = 16.14$; $p < 0.001$). The prevalence rate of BTV in *C. variipennis* was significantly for the calf than the sheep at the Ausman farm ($p = 0.008$), but did not differ statistically at the Fields farm ($p = 0.628$).

5. BTV prevalence in other *Culicoides* species

The MFIR's for other *Culicoides* species were 6.5% (13/200) for *C. crepuscularis* at the Fields site and 12.22% (11/90) at the Ausman site. Thus the prevalence rate of BTV in *C. crepuscularis* did not differ statistically ($X^2 = 1.7$; $p > 0.1$). For *C. haematopodus* the MFIR's for the Fields site was 4.16% (1/24) and for the Ausman site 12.5% (4/32). Thus the prevalence rate of BTV in *C. haematopodus* did not differ statistically ($X^2 = 1.17$; $p > 0.1$). The MFIR for the *C. cockerellii* at the Fields farm site was 0% (0/9).

D. Discussion

BTV activity has been documented for the past 10 years in endemic areas of Colorado. These areas contain large

numbers of *Culicoides* species. *C. variipennis* had been the most studied because of its role in the transmission of BTV. Major determinants of vectoral capacity of *C. variipennis* for BTV include host preferences and temporal feeding activity. Drop traps utilizing selected bait animals have been used to collect engorged *Culicoides* to investigate these determinants.

During this study, 11,466 insects were collected, and sorted by date, farm site, animal, time period, species, and blood fed versus non-blood fed. Blood feeding was greatest at the Fields site in September and at the Ausman site in July, when the heaviest insect attack rate occurred per animal, and high numbers of *C. variipennis* were recovered. This discrepancy could be explained by the fact that collections were stopped at the Ausman site after August 19 due to personnel shortages and time constraints. If collections had continued at this site, an increase comparable to that seen at the Fields site might have been observed. The calf consistently attracted greater numbers of insects than the sheep. The exception was *C. crepuscularis*, which preferred the sheep to the calf. Sheep have been reported previously to not be as attractive to *C. variipennis* for blood feeding as cattle (DuToit, 1962). Clearly, in this study, the *C. variipennis* preferred to feed on cattle, even when sheep were in close proximity. Since

Culicoides are responsive to CO₂, cattle, which exhale large amounts of CO₂, may be more attractive than sheep.

Time period 1, at the Fields site and time period 4 at the Ausman site were time periods for maximum insect activity. This discrepancy cannot be easily explained. The Ausman site is approximately 20 miles to the south of the Fields site and air temperatures may remain warmer after sunset at this farm site. Another factor to take into account would be the separate team of investigators working at each site. Perhaps, there was an inherent bias in the aspiration of insects at each site. Statistical analyses (t tests) did not show a significant difference between the mean *C. variipennis* captures from the calf and sheep at either farm site for periods 1-5. There were, however, significant differences between the mean *C. variipennis* captures from the calf or sheep at the Ausman farm for periods 2, 3, and 4 and from the Fields farm for periods 1 and 3.

BTV prevalence in insects was determined by BTV antigen-capture ELISA. The MFIR's of *C. variipennis* in the study areas averaged 4.5% at the Fields site and 11% at the Ausman site. This difference is significant ($p < 0.001$). It is especially of interest to note that the MFIR of *C. crepuscularis* was 7.5% at the Fields site and 12.22% at the Ausman site ($p > 0.1$). The MFIR of *C. haematopodus* ranged

from 4% at the Fields site to 12.5% at the Ausman site ($p>0.1$). These two insects may play an as yet unknown but important role in BTV transmission in these study areas.

Detecting virus antigen in adult *Culicoides* of other species, however, does not necessarily mean that these species are vectors of BTV. Any blood-sucking arthropod feeding on viremic blood can retain an arbovirus for a short period of time. Nevill and Anderson (1972) reported that more than one *Culicoides* species may be involved in the transmission of BTV and concluded that in South Africa, both *C. pallidipennis* and *C. schultzei* may be involved. Therefore, further investigation of these *Culicoides* species needs to be done, especially in endemic farm sites such as the ones described in this study. One possible hypothesis is that while *C. variipennis* is the established epidemic vector of BTV, another species of *Culicoides* could act as the endemic vector of BTV.



Figure 3.1A. Drop trap apparatus at the Fields farm.



Figure 3.1B. Drop trap apparatus at the Ausman farm.



Figure 3.2A. Study animals being brushed to dislodge insects.



Figure 3.2B. Exposure of animals to the open air.



Figure 3.2C. Cages lowered to allow complete engorgement of the insects (animals were removed for the picture).



Figure 3.2D. Vacuum aspirator for insects.

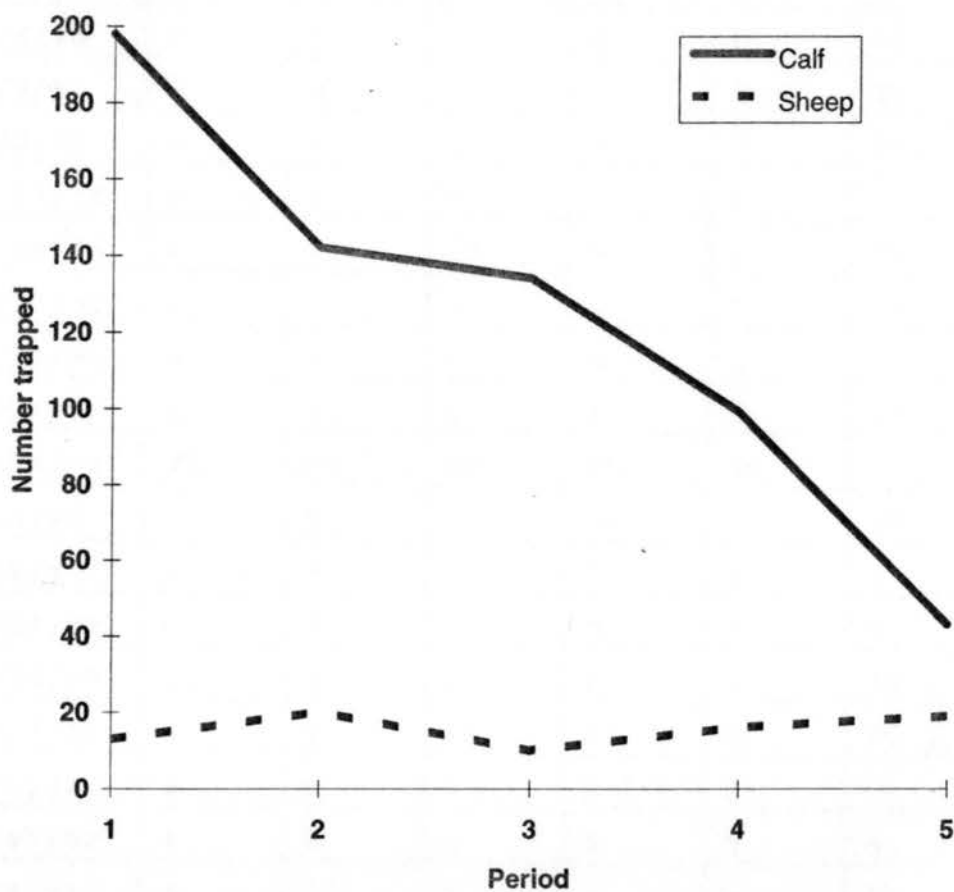


Figure 3.3. Fields site-Total *C. variipennis* trapped per period.

Table 3.1. *Culicoides variipennis* captured from the Fields (F) farm site from the calf (C) during periods 1-5 on each date tested.

Date	FC1	FC2	FC3	FC4	FC5	Total
6/29/92	16	8	17	18	17	76
7/1/92	6	5	4	6	0	21
7/6/92	2	28	4	12	0	36
7/8/92	9	6	1	5	0	21
7/13/92	4	1	5	0	1	11
7/15/92	4	10	11	3	0	28
7/20/92	9	7	1	1	1	19
7/22/92	21	6	2	0	0	30
7/27/92	4	10	9	4	3	30
7/29/92	ND	ND	ND	ND	ND	ND
8/3/92	0	0	3	14	3	20
8/5/92	2	7	1	0	7	17
8/10/92	0	0	0	0	0	0
8/12/92	21	2	4	0	1	28
8/17/92	9	5	20	6	0	40
8/19/92	0	0	4	3	2	9
8/27/92	4	2	0	0	0	6
9/2/92	0	13	7	4	0	24
9/10/92	67	21	33	13	2	136
9/17/92	0	18	2	10	6	36
9/22/92	20	3	5	0	0	28
Total	198	142	134	99	43	616

ND = Not done on this collection date

F = Fields farm site

C = calf

Table 3.2. *Culicoides variipennis* captured from the Fields (F) farm site from the sheep (s) during periods 1-5 on each date tested.

Date	FS1	FS2	FS3	FS4	FS5	Total
6/29/92	1	1	1	0	0	3
7/1/92	0	0	0	0	1	1
7/6/92	0	2	0	2	10	14
7/8/92	3	1	0	1	0	5
7/13/92	0	0	0	0	0	0
7/15/92	0	7	0	0	0	7
7/20/92	1	1	2	0	0	4
7/22/92	2	0	0	0	0	2
7/27/92	0	0	0	1	1	2
7/29/92	ND	ND	ND	ND	ND	ND
8/3/92	1	0	1	1	2	5
8/5/92	1	0	0	0	1	2
8/10/92	0	0	0	1	0	1
8/12/92	0	0	0	0	1	1
8/17/92	0	0	1	1	0	2
8/19/92	0	0	3	4	3	10
8/27/92	0	0	0	1	0	1
9/2/92	0	1	1	0	0	2
9/10/92	3	5	1	4	0	13
9/17/92	0	0	0	0	0	0
9/22/92	1	2	1	0	0	3
Total	13	20	10	16	19	78

ND = Not done on this collection date

F = Fields farm site

S = sheep

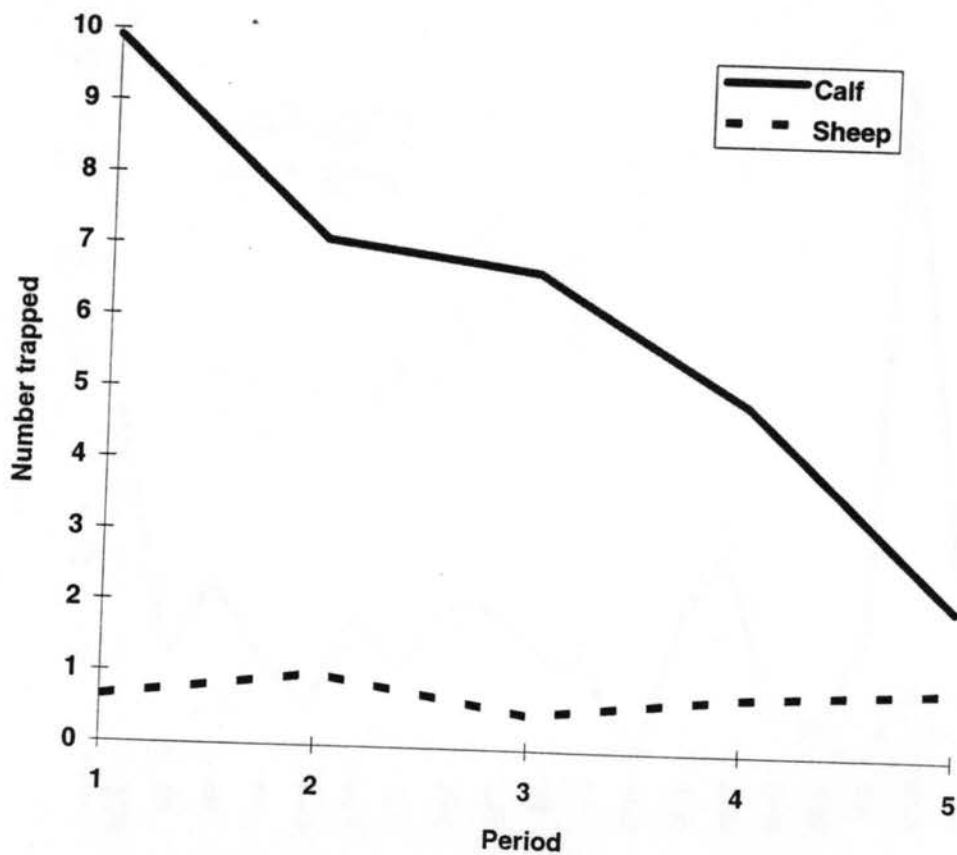


Figure 3.4. Fields site-Nightly period average of *C. variipennis* trapped.

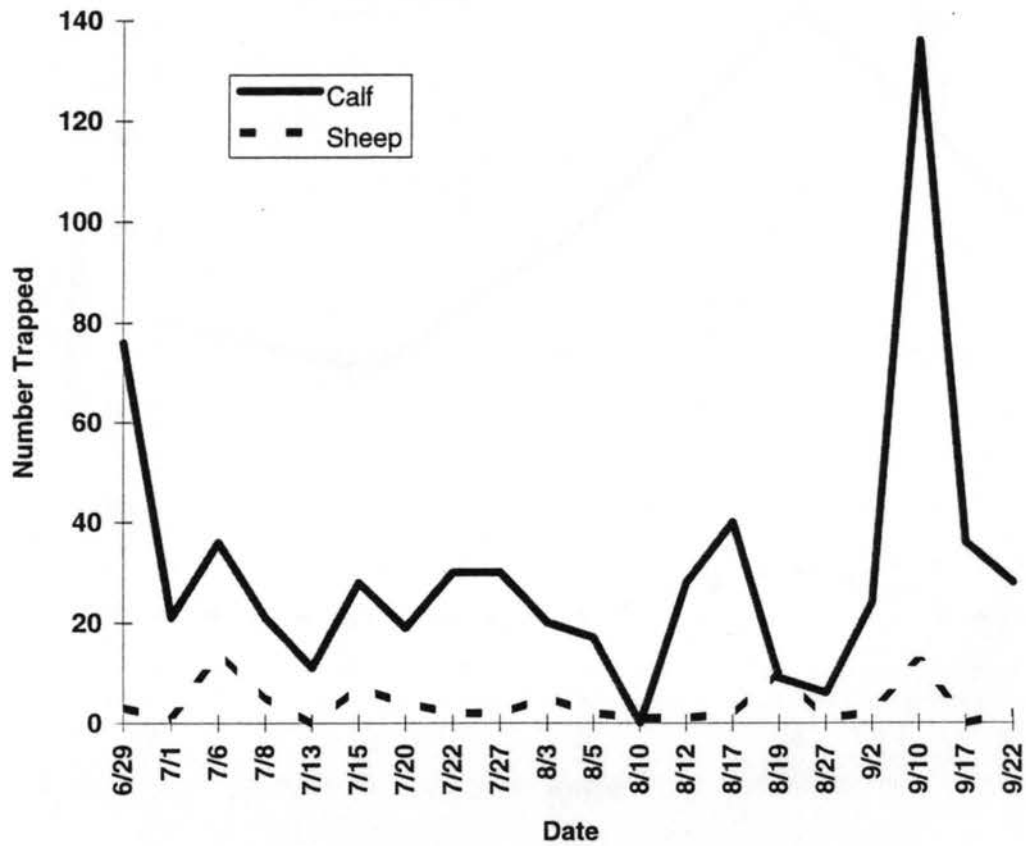


Figure 3.5. Fields site-Total *C. variipennis* per trap date.

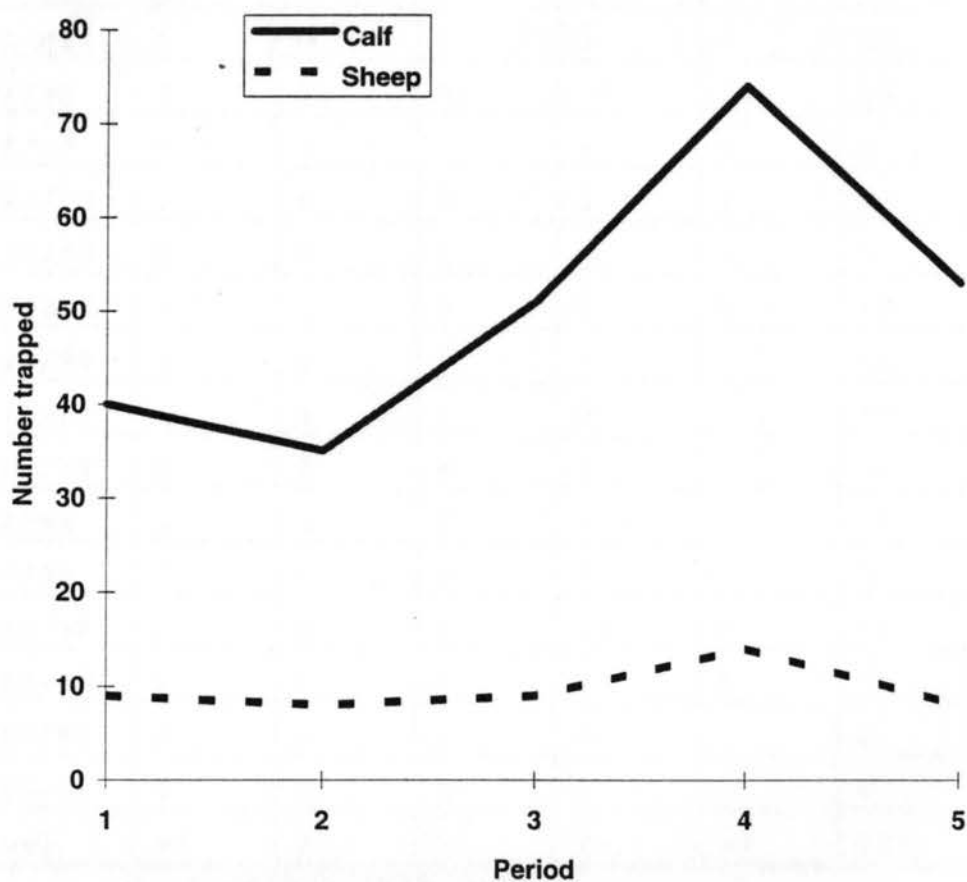


Figure 3.6. Ausman site-Total *C. variipennis* trapped per period.

Table 3.3. *Culicoides variipennis* captured from the Ausman (A) farm site from the calf (C) during periods 1-5 on each date tested.

Date	AC1	AC2	AC3	AC4	AC5	Total
6/29/92	0	0	2	2	0	2
7/1/92	9	0	1	2	0	10
7/6/92	6	10	18	38	23	95
7/8/92	0	0	0	0	0	0
7/13/92	9	6	0	1	1	17
7/15/92	0	0	0	0	3	3
7/20/92	1	0	3	6	0	10
7/22/92	8	1	0	5	1	15
7/27/92	4	1	1	0	4	10
7/29/92	0	2	8	0	5	15
8/3/92	1	1	7	9	0	18
8/5/92	0	1	0	0	1	2
8/10/92	0	9	5	13	9	36
8/12/92	0	3	1	1	5	10
8/17/92	1	0	2	0	1	4
8/19/92	1	1	3	1	0	6
Total	40	35	51	74	53	253

A = Ausman farm site

C = calf

Table 3.4. *Culicoides variipennis* captured from the Ausman (A) farm site from the sheep (S) during periods 1-5 on each date tested.

Date	AS1	AS2	AS3	AS4	AS5	Total
6/29/92	0	0	1	0	0	1
7/1/92	1	0	0	0	0	1
7/6/92	1	5	4	3	2	15
7/8/92	0	0	0	0	0	0
7/13/92	1	0	0	2	0	3
7/15/92	0	0	0	0	2	2
7/20/92	1	0	1	2	0	4
7/22/92	0	0	0	0	0	0
7/27/92	2	0	0	1	2	5
7/29/92	2	0	1	2	1	6
8/3/92	0	3	2	0	0	5
8/5/92	0	0	0	0	0	0
8/10/92	0	0	0	2	1	3
8/12/92	0	0	0	0	0	0
8/17/92	0	0	0	0	0	0
8/19/92	1	0	0	2	0	3
Total	9	8	9	14	8	48

A = Ausman farm site

S = sheep

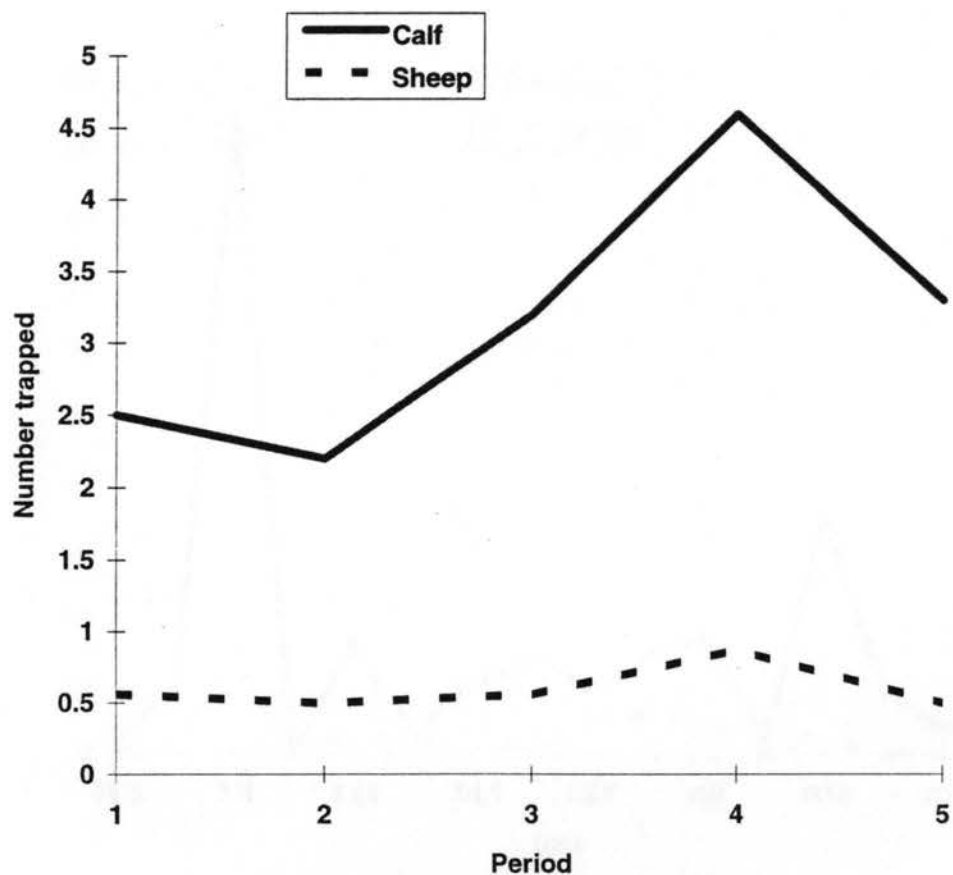


Figure 3.7. Ausman site-Nightly period average of *C. variipennis* trapped.

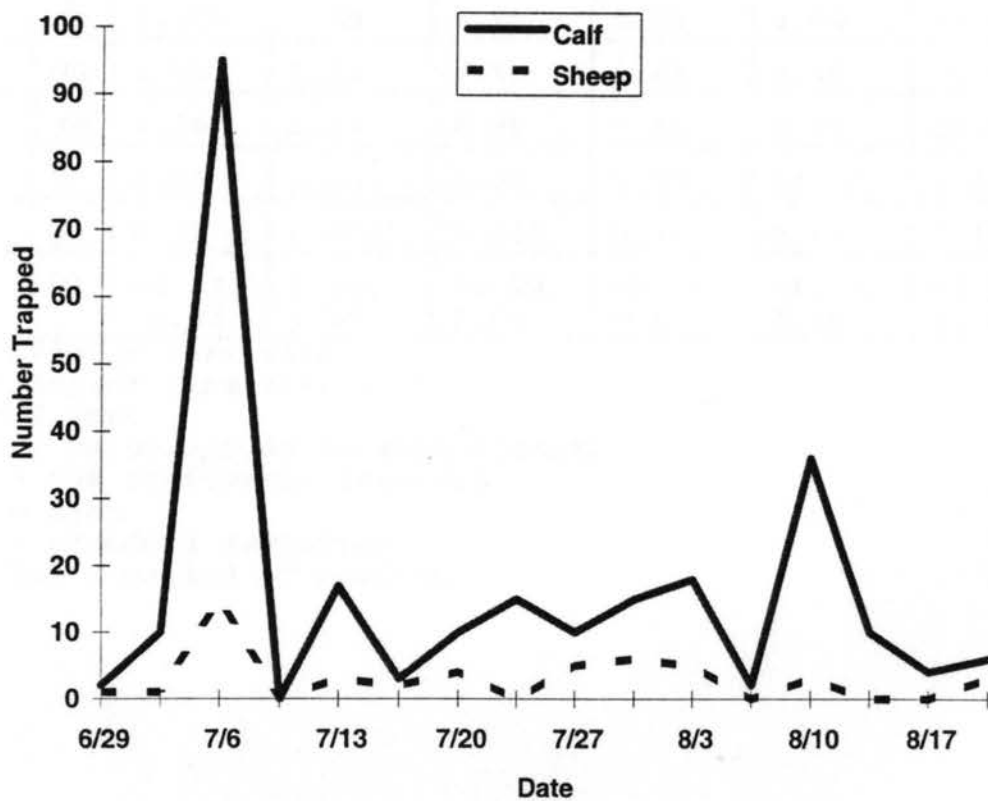


Figure 3.8. Ausman site-Total *C. variipennis* per trap date.

Table 3.5. Statistical analysis of the mean *C. variipennis* captures from the calf for periods 1 to 5 from the Fields and Ausman farm sites during the study.

		Period					
		1	2	3	4	5	Total
F	Mn	9.90	7.60	6.65	4.95	2.15	30.8
	SD	15.20	7.49	8.20	5.58	4.04	29.40
A	Mn	2.50	2.19	3.19	4.87	3.31	15.80
	SD	3.48	3.25	4.69	9.61	5.85	22.90
	T	1.90	2.69	1.50	0.03	-0.70	1.67
	p	0.07	0.011	0.014	0.98	0.49	0.10
	CI	-0.50, 16.33	1.30, 9.50	-1.20, 8.10	-5.10, 5.30	-4.52, 2.20	-3.20, 33.20

F = Fields farm site

A = Ausman farm site

T = T test

p = p value (≤ 0.05 is significant)

CI = 95% confidence interval

Mn = mean

SD = standard deviation

Period = period of testing

Table 3.6. Statistical analysis of the mean *C. variipennis* captures from the sheep for periods 1 to 5 for the Fields and Ausman farm sites during the study.

		Period					
		1	2	3	4	5	Total
F	Mn	0.65	1.00	0.55	0.80	0.90	3.90
	SD	0.99	1.86	0.83	1.24	2.25	4.09
A	Mn	0.56	0.50	0.56	0.87	0.50	3.00
	SD	0.73	1.41	1.09	1.09	0.82	3.79
	T	0.30	0.89	-0.04	-0.19	0.68	0.68
	p	0.77	0.38	0.97	0.85	0.50	0.50
	CI	-0.51, 0.69	-0.65, 1.65	-0.66, 0.64	-0.88, 0.73	-0.80, 1.60	-1.80, 3.60

F = Fields farm site

A = Ausman farm site

T = T test

p = p value (≤ 0.05 is significant)

CI = 95% confidence interval

Mn = mean

SD = standard deviation

Period = period of testing

Table 3.7. Statistical analysis of the mean *C. variipennis* captures from the Ausman farm site for periods 1 to 5 from the calf and the sheep during the study.

		Period					
		1	2	3	4	5	Total
C	Mn	9.90	7.60	6.65	4.95	2.15	30.80
	SD	15.20	7.49	8.20	5.58	4.04	29.40
S	Mn	0.65	1.00	0.55	0.80	0.90	3.90
	SD	0.99	1.86	0.83	1.24	2.25	4.09
	T	2.71	3.83	3.31	3.25	1.21	4.06
	p	0.10	0.0005	0.0021	0.0024	0.23	0.0002
	CI	2.30, 16.17	3.10, 10.09	2.40, 9.83	1.60, 6.74	-0.84, 3.34	13.50, 40.33

C = calf

S = sheep

T = T test

p = p value (≤ 0.05 is significant)

CI = 95% confidence interval

Mn = mean

SD = standard deviation

Period = period of testing

Table 3.8. Statistical analysis of the mean *C. variipennis* captures from the Fields farm site for periods 1 to 5 from the calf and the sheep during the study.

		Period					
		1	2	3	4	5	Total
C	Mn	2.50	2.19	3.19	4.87	3.31	15.80
	SD	3.48	3.25	4.68	9.61	5.85	22.90
S	Mn	0.56	0.50	0.56	0.87	0.50	3.00
	SD	0.73	1.41	1.09	1.09	0.82	3.79
	T	2.18	1.90	2.19	1.65	1.90	2.21
	p	0.04	0.07	0.04	0.11	0.07	0.04
	CI	0.12, 3.75	-0.12, 3.50	0.20, 5.08	-0.90, 8.94	-0.20, 5.83	1.00, 24.66

C = calf

S = sheep

T = T test

p = p value (≤ 0.05 is significant)

CI = 95% confidence interval

Mn = mean

SD = standard deviation

Period = period of testing

IV. ANALYSIS OF *CULICOIDES* SPECIES GENOMIC FINGERPRINTS USING tRNA GENE PRIMERS IN A POLYMERASE CHAIN REACTION

A. Introduction

The genus *Culicoides* Latreille (Diptera: Ceratopogonidae) consists of over 4000 species worldwide (Wirth et al. 1980), many of which have been identified on the basis of wing pattern and adult size. The biting midge, *Culicoides variipennis* (Coquillett), is the primary North American vector of BTV, which causes disease in sheep, cattle, and ruminant wildlife. Other species of *Culicoides* vector BTV in Africa, Australia, Eastern Asia, and the Mediterranean (Walton & Osburn 1992). Five subspecies of *C. variipennis* have been recognized in the U. S. (Wirth & Jones 1957): *C. v. albertensis* (midwest U. S.), *C. v. australis* (southeast U. S.), *C. v. occidentalis* (west and Pacific coast U. S.), *C. v. sonorensis* (southwest U. S.), and *C. v. variipennis* (northeast U. S.). In each region of the world the accurate identification of proven or potential vectors has proved difficult (Tabachnick et al. 1992). This is evident in identifying members of the "*variipennis*" complex (Wirth & Morris 1985). The existence of three of these subspecies, *C. v. occidentalis*, *C. v. sonorensis*, and *C. v.*

variipennis, has been confirmed using laborious isozyme electrophoretic techniques (Tabachnick, 1990, 1992). In addition, present taxonomy of *Culicoides* almost exclusively relies on adult characteristics, such as wing pattern identifications. There are 248 valid species and subspecies of *Culicoides* with wing patterns ranging from clear to multi-spotted. Species identifications using larval and pupal stages have been described but are extremely difficult, requiring considerable skill in insect morphology (Murphree & Mullen, 1991). Also, many larvae and pupae die before emergence as adults when placed into a laboratory setting, resulting in a time constraint on the identification process. Species identification, however, is necessary to identify possible vector populations which would allow the early implementation of effective vector control strategies. If BTV is only transmitted by one vector in a certain area, accurate species identification at the larval level eliminates the expense of chemically treating breeding sites colonized by harmless species of *Culicoides*.

The polymerase chain reaction (PCR) has been very useful for diagnosis of pathogens and for molecular taxonomy and genetic polymorphism analysis (Bloch 1991). PCR is a technique in which a specific sequence of the target DNA is amplified exponentially during a specific set of cycles.

Each cycle consists of first denaturing the DNA, annealing the corresponding primers, and extending the primers across the templates with DNA polymerase. Amplified sequences are then separated electrophoretically on an ethidium-stained agarose gel and detected visually with ultra-violet light. Further steps can be taken such as southern blot hybridization to increase the detection specificity. Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) has been used to detect DNA polymorphisms in aphids and mosquitoes using small, 10-12 base, random primers (Kambhampati et al. 1992, Black et al. 1992). Ribosomal RNA (rRNA) has been used as the target sequence in PCR, and by using 16s rRNA-specific probes, organisms can be distinguished on a kingdom, genus, species, or strain specific level (Pickup 1991). The arbitrarily-primed polymerase chain reaction (AP-PCR) generates reproducible fingerprints that include DNA fragment polymorphisms, which can be used to distinguish between strains of organisms (Welsh et al., 1991; Welsh and McClelland, 1990).

Transfer RNA (tRNA) genes, like rRNA genes, occur in multiple copies throughout the genome of most organisms. Playing an essential role in translation, the tRNA genes have been studied extensively in *Escherichia coli* and *Bacillus subtilis* (Giroux et al., 1988). The tRNA genes in bacterial genomes are generally arranged in a head to tail

fashion, tend to be clustered, and may be organized into operons. One operon in *P. phosphoreum* has eight genes and five pseudogenes in a region of less than 1500 bp (Giroux et al., 1988). In the human genome there are 1300 tRNA genes and a large number of tRNA pseudogenes (Welsh and McClelland, 1991). Because the tRNA genes share some sequence homology, primers for PCR that contain consensus tRNA sequences are likely to result in a number of characteristic "fingerprint" banding patterns. These fingerprint patterns might be relatively stable over evolutionary time scales, since the organization of tRNA genes is expected to evolve relatively slowly. The banding patterns are presumably derived from the amplification of regions between closely-linked tRNA genes. Consensus tRNA gene primers have been used to differentiate strains of *Staphylococcus* and *Streptococcus* at the species level in a PCR assay (Welsh & McClelland 1991).

The research hypothesis for this study was that consensus tRNA gene primers can be used to differentiate the *Culicoides* species commonly found in Colorado. These primers should be able to differentiate the *Culicoides* species regardless of the life cycle stage of the midge. This aspect would help in identifying *Culicoides* at larval breeding sites before they reach the adult stage. Mixed or heterogeneous pools of *Culicoides* species must be

differentiated and not confused with homogeneous pools of one *Culicoides* species. To test the hypothesis, the usefulness of these tRNA primers as taxonomic tools in differentiating *Culicoides* species as adults, pupae, and larvae was investigated.

B. Materials and Methods

1. Collection of field *Culicoides* species

Field specimens of *C. variipennis*, *C. haematopodus* Malloch, *C. crepuscularis* Malloch, and *C. cockerellii* (Coquillett), were collected from two premises in Weld and Adams counties of Northern Colorado during the summer of 1991. The Ausman farm was located near Brighton, Colorado; the Fields farm was located approximately 20 miles to the north. The insects were collected in New Jersey light traps using a 40 watt light source, stored in ethanol, and sorted by species using adult wing identification patterns (Fig. 4.1). The specimens were then air dried and tested by tRNA-PCR.

2. Control *C. variipennis*

Control *C. v. sonorensis* adults, pupae, and larvae were from the AA colony (Sonora strain), which has been maintained in the laboratory since 1957 (Jones 1960) and were kindly provided by the USDA-ABADRL. Rearing and maintenance of the flies were as described by Jones et al.

(1969). The control insects were also stored in ethanol and air dried before testing.

3. DNA extraction

Culicoides adults were triturated in 300 μ l of a lysis buffer containing 20 mg/ml proteinase K, 100mM Tris-HCl, pH 8.0, 50 mM NaCl, 50 mM EDTA, 1% SDS, 0.15 mM spermine, and 0.5 mM spermidine, and then incubated overnight at 50°C. A phenol extraction step was followed by an ethanol precipitation, and the DNA was resuspended in 20 μ l of water and incubated at 50°C for 2 hours. Two μ l of each sample were used for the tRNA PCR homogeneous reactions, while 1 μ l of each sample was used for the tRNA-PCR heterogeneous reactions.

4. tRNA-PCR procedure

Two consensus tRNA gene primers, T₅A and T₃A, were investigated. Given that there are many divergent tRNA genes in a typical genome, primers were produced that 1) have at least a five base perfect match between the 3' end of the primer and many of the tRNA genes and 2) have extensive homology in the rest of the primer with a number of different tRNA genes from a wide variety of sources (Welsh and McClelland, 1991). The T₅A primer (5'AGTCCGGTGCTCTAACCAACTGAG 3') was derived from the complement of the consensus sequences at the 5' end of tRNA genes and faces out from the gene in the 5' to 3' direction;

the T₃A primer (5' GGGGGTTCGAATTCCCGCCGGCCCCA 3') is a consensus sequence at the 3' end facing out of the genes in the 5' to 3' direction (Fig. 4.2). The tRNA-PCR contained the four dNTPs (0.1 mM each), 20 mM MgCl₂, 100 mM Tris-HCl (pH 8.3), 100 mM KCl, 0.01% gelatin, Taq DNA polymerase (0.5 U), and the primer T₃A or T₅A (15 ng). Cycles were 94°C for 4 min once, then 94°C for 30 sec, 50°C for 30 sec, and 72°C for 2 min, for 45 cycles. The samples were stored at 4°C until electrophoresis using a 1.4% agarose gel in Tris-borate-EDTA (TBE) buffer for four hours at 5V/cm, and staining with ethidium bromide (0.5 ug/ml).

C. RESULTS

Both tRNA primers produced unique banding patterns in the different species, but T₃A was more useful and only its results are presented. Results of PCR of the 4 species tested (Fig. 4.3) showed unique bands at 1, 2.6, 1.2, and 0.8 kb for *C. variipennis*, *C. haematopotus*, *C. crepuscularis*, and *C. cockerellii*, respectively. *Culicoides v. sonorensis* laboratory adults and pupae were also tested and they also showed a unique band at 1 kb similar to the field collected adult *C. variipennis*.

When heterogeneous pools of DNA composed of two or even three different species were tested, each species could be differentiated from the other species by its unique banding pattern (Fig. 4.4). When *C. haematopotus*, *C. crepuscularis*,

and *C. variipennis* adult samples were mixed, the respective unique bands for each species at 1, 2.6, and 1.2 kb, could be distinguished (Table 4.1).

Table 4.1. Unique banding patterns derived from heterogeneous pools of DNA tested from several species of *Culicoides*

Species	Corresponding unique band			
	2.6Kb	1.2Kb	1.0Kb	0.8Kb
<i>C. haematopodus</i> (<i>C. haem</i>)	XX			
<i>C. crepuscularis</i> (<i>C. crep</i>)		XX		
<i>C. cockerellii</i> (<i>C. cock</i>)				XX
<i>C. variipennis</i> (<i>C. var</i>)			XX	
<i>C. haem</i> + <i>C. crep</i>	XX	XX		
<i>C. haem</i> + <i>C. cock</i>	XX			XX
<i>C. haem</i> + <i>C. var</i>	XX		XX	
<i>C. crep</i> + <i>C. cock</i>		XX		XX
<i>C. crep</i> + <i>C. var</i>		XX	XX	
<i>C. var</i> + <i>C. cock</i>			XX	XX
<i>C. haem</i> + <i>C. crep.</i> + <i>C. var</i>	XX	XX	XX	

The tRNA-PCR of different life stages (including adult, pupal, and larval) of *C. variipennis* revealed two unique bands of 1.0 and 1.3 kb (Fig. 4.5).

Previously unidentified field larvae collected near Brighton, Colorado were tested and approximately 50% could be positively identified as *C. variipennis*. Fig. 4.6 shows that lanes 2-6, 8-11, 13, and 21 share the unique 1 kb fragment similar to both field-collected and laboratory colony *C. variipennis* adults.

D. DISCUSSION

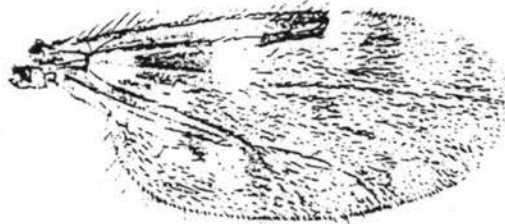
PCR, using only a single consensus tRNA gene primer, appears to be a promising tool in differentiating the four species of *Culicoides* found in Northern Colorado. The technique is capable of identifying species, regardless of life stage. Additional studies will have to be performed with field collected material to determine the applicability of the technique to differentiation of these and related species from other geographic locales.

A major advantage of the tRNA-PCR technique is that no prior sequence information is needed to generate primers. Also, only small amounts of the individual insect are needed to isolate DNA; therefore, the rest of the sample could be saved for evaluating other taxonomic criteria or for isolating pathogens. Future studies will include investigating the usefulness of these techniques in differentiating *C. variipennis* complex subspecies and further larval taxonomic studies. The use of PCR in taxonomic investigations will continue to grow and these

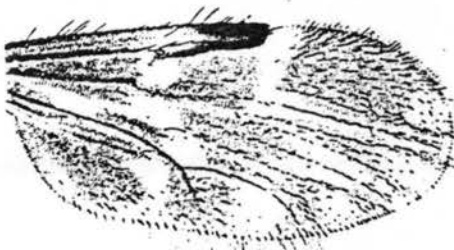
simple tools will become invaluable in vector surveillance and control operations by providing species and population genetic markers.



C. varilpennis



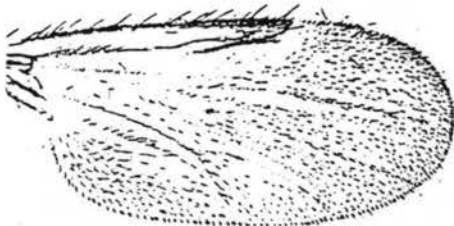
C. crepuscularis



C. haematopodus



C. stellifer



Seiffia group



C. cockerelli

Figure 4.1. Adult wing patterns of *Culicoides* species commonly found in Colorado. Field identifications of *Culicoides* species depend on adult wing identifications. There are approximately 248 valid species and subspecies of *Culicoides* with wing patterns ranging from clear to multi-spotted. The wing patterns of the four species used in this study are shown above (Blanton and Wirth, 1979) as well as two related species.

T₃A 5' AGTCCGGTGCTCTAACCAACTGAG 3'

T₅A 5' GGGGGTTCGAATTCCCGCCGGCCCCA 3'

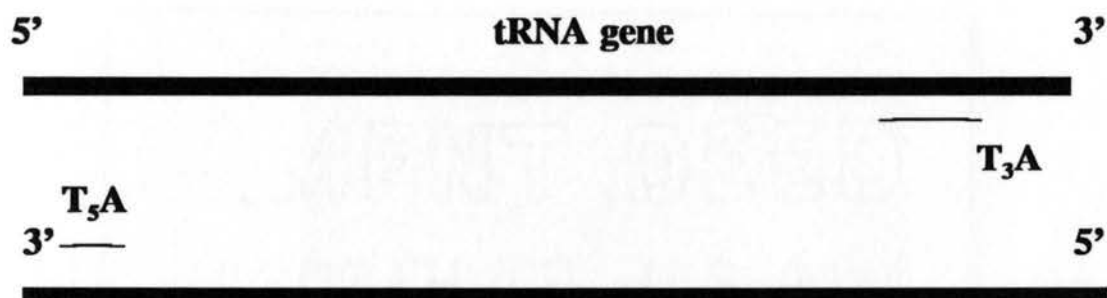


Figure 4.2. Primers used in the tRNA PCR assay. Two consensus tRNA gene primers, T₅A and T₃A, were investigated as to their possible use as taxonomic tools. The T₅A primer was derived from the complement of the consensus sequence at the 5' end of the tRNA genes and faces out from the gene in the 5' to 3' direction. The T₃A primer is a consensus sequence at the 3' end facing out of the genes in the 5' to 3' direction. Both primers generated unique banding patterns, however T₃A showed to be the most useful and the following experiments use T₃A only.

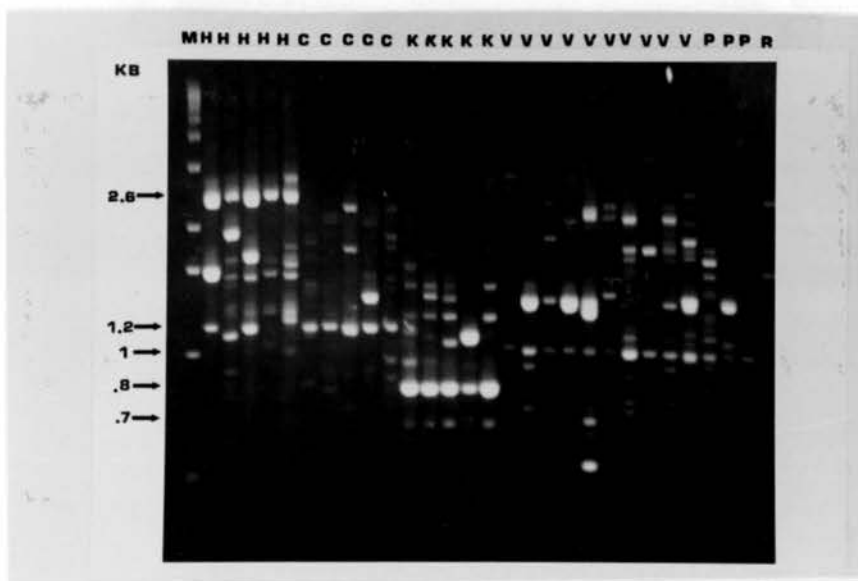


Figure 4.3. Electrophoretic comparison of field-collected and laboratory colony *Culicoides* species. Adult and pupal stage flies were tested by tRNA PCR and run on a 1.4% agarose gel stained with ethidium bromide. Lane 1, 1 Kb DNA ladder marker; Lanes 2-6, *Culicoides haematopodus* field adults; Lanes 7-11, *Culicoides crepuscularis* field adults; Lanes 12-16, *Culicoides cockerellii* field adults; Lanes 17-21, *Culicoides variipennis* field adults; Lanes 22-26, *Culicoides variipennis* laboratory adults; Lanes 27-29, *Culicoides variipennis* laboratory pupae; Lane 30, Control T₃A primer only.

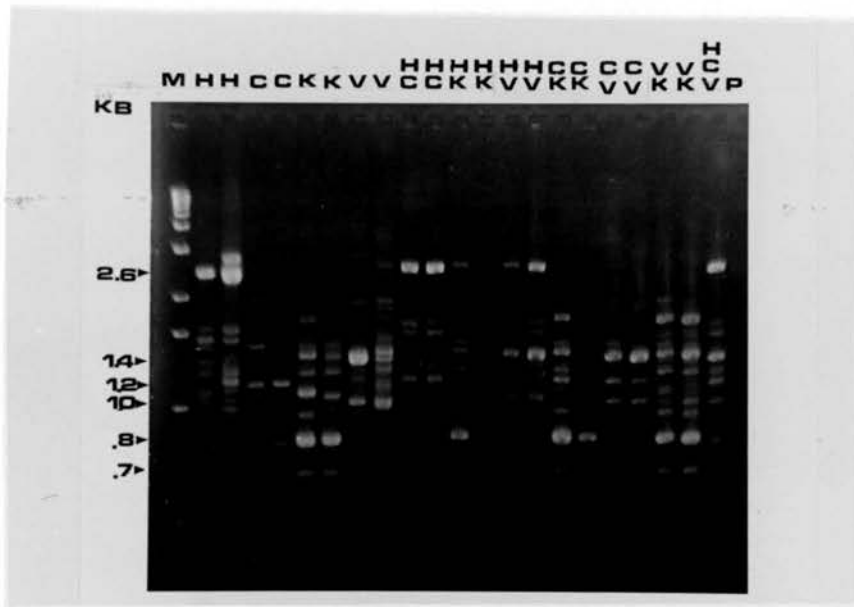


Figure 4.4. Electrophoretic comparison of heterogeneous mixtures of field-collected and laboratory colony *Culicoides* species. Adult and pupal stage flies were tested by tRNA PCR and run on a 1.4% agarose gel stained with ethidium bromide. Lane 1, 1 Kb DNA ladder marker; Lanes 2-3, *Culicoides haematopodus* adults; Lanes 4-5, *Culicoides crepuscularis* adults; Lanes 6-7, *Culicoides cockerellii* adults; Lanes 8-9, *Culicoides variipennis* laboratory adults; Lanes 10-11, *C. haematopodus*/*C. crepuscularis* mixed adults; Lanes 12-13, *C. haematopodus*/*C. cockerellii* mixed adults; Lanes 14-15, *C. haematopodus*/*C. variipennis* mixed adults; Lanes 16-17, *C. crepuscularis*/*C. cockerellii* mixed adults; Lanes 18-19, *C. crepuscularis*/*C. variipennis* mixed adults; Lanes 20-21, *C. variipennis*/*C. cockerellii* mixed adults; Lane 22, *C. haematopodus*/*C. crepuscularis*/*C. variipennis*; Lane 23, Control T₃A primer only.

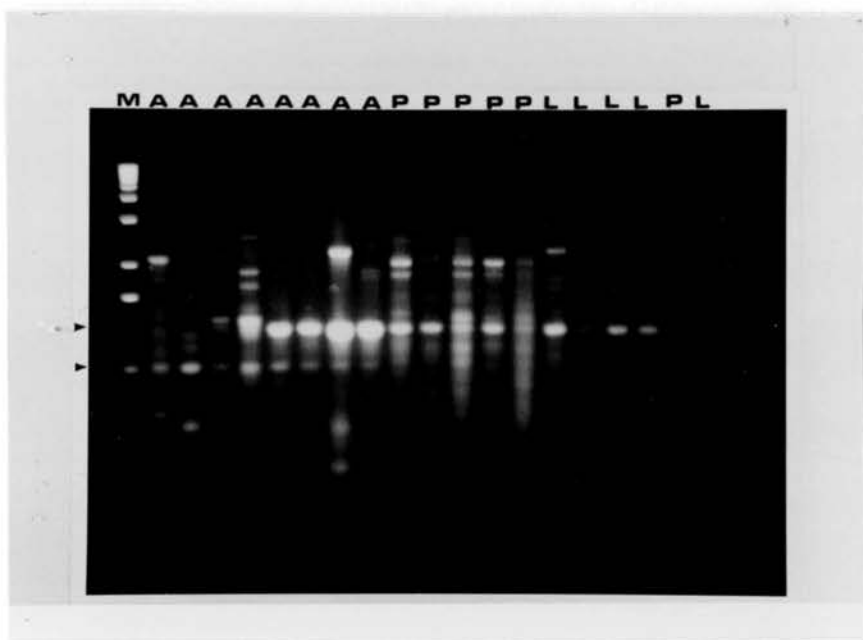


Figure 4.5. Electrophoretic comparison of adult, pupal, and larval stages of *Culicoides variipennis* laboratory colony. Adult, pupal and larvae stage flies were tested by tRNA PCR and run on a 1.4% agarose gel stained with ethidium bromide. Lane 1, 1 Kb DNA ladder marker; Lanes 2-5, *C. variipennis* field adults; Lanes 6-9, *C. variipennis* laboratory adults; Lanes 10-14, *C. variipennis* laboratory pupae; Lanes 15-18, *C. variipennis* laboratory larvae; Lane 19, Control T₃A primer only; Lane 20, *C. variipennis* laboratory larvae.

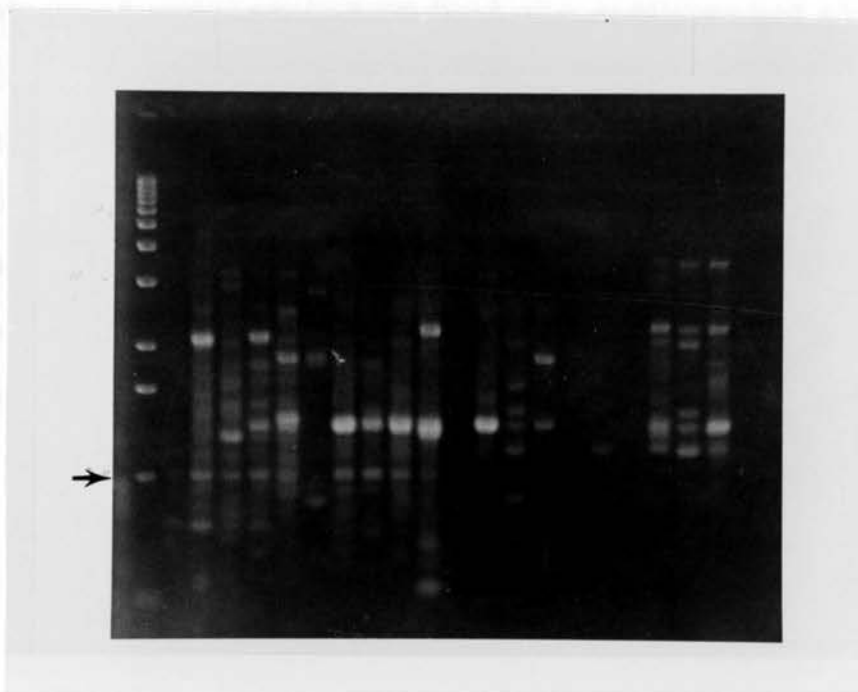


Figure 4.6. Electrophoretic comparison of random unidentified field collected larvae from the Ausman farm near Brighton, Colorado. Larvae were tested by tRNA PCR and run on a 1.4% agarose gel stained with ethidium bromide. Lane 1, 1 Kb DNA ladder marker; Lanes 2-21, Random field larvae from Ausman farm; Lane 22, Control T₃A primer only. The arrow indicates the 1.0 Kb unique band found for *C. variipennis*.

V. DEVELOPMENT OF A REVERSIBLE TARGET CAPTURE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RTC-RT-PCR) FOR THE DETECTION OF BLUETONGUE VIRUS

A. Introduction

Currently used procedures for diagnosis of BTV infections and for BTV surveillance are inadequate. Virus isolation is problematic in that the embryonated chicken egg remains the most sensitive host, and it is a laborious, time-consuming and expensive assay for the diagnosis of BTV. Serological diagnosis relies on agar gel immunodiffusion (AGID), serum neutralization, or ELISA techniques, which, while faster, do not allow direct detection of the virus. Nucleic acid hybridization with a labeled probe has been used in many applications to detect target sequences in samples of interest (Wetmur, 1991). Blot and sandwich hybridization assays have been developed (Schoepp et al, 1990) to rapidly detect BTV. However, these often require tedious sample preparation protocols, have slow reaction kinetics, and low sensitivities for clinical samples.

Solution hybridization formats using cell lysates solubilized in guanidine isothiocyanate have been used to identify BTV and other RNA viruses (Dangler et al, 1988, Thompson and Gillespie, 1987, Pelligrino et al, 1987).

Chaotropic salts, like guanidinium thiocyanate (GuSCN), in concentrations over 4M lower the melting temperature (T_m) of DNA (Von Hippel and Wong, 1964). GuSCN lyses cells, inhibits nucleases and proteases, but does not chemically alter the viral nucleic acid analyte (Van Ness and Chen, 1991). Hybridization reactions have been described that utilize covalent binding of nucleic acids to magnetic beads (Lund et al, 1988, Uhlen, 1989) and sephacryl beads (Kwoh et al, 1989). However, these often employ a radiolabeled probe, which is a major drawback to clinical diagnostic techniques due to personnel safety concerns, waste disposal problems, and the short half-life of many radioisotopes.

The reversible target capture (RTC) assay detects specific nucleic acid sequences in crude cell lysates utilizing varying concentrations of GuSCN in a hybridization assay (Thompson et al, 1989a, 1989b). The RTC was originally developed by Morrissey et al (1989a, 1989b) as a general technique to make molecular hybridization assays more attractive to clinical laboratories. In the RTC assay, a complex is initially formed in concentrated solutions of guanidine thiocyanate consisting of the viral RNA analyte, the labeled detector probe, and capture probe. The capture probe is a 3' poly (dA) tailed oligonucleotide complementary to the analyte and either upstream or downstream in sequence from the detector probe. The detector probe can be labeled

radioactively with ^{32}P or nonradioactively with digoxigenin. The ternary complex is then "captured" by oligo (dT) derivitized paramagnetic beads by allowing the capture probe to form a bridge between the ternary complex and the oligo (dT) beads. This bridge forms the weakest hybrid in the assay. After the excess unhybridized probe and debris are washed away, the concentration of guanidine thiocyanate in the hybridization medium is raised, selectively breaking this bridge and leaving the ternary complex intact. After elution, the ternary complex can be "captured" again on poly dT filters. By using high concentrations of the capture probe and oligo (dT) beads, the hybridization reaction is driven so that capture of the ternary complex occurs within minutes. Multiple captures reduce the background due to non-specific binding of detector probe to virtually undetectable levels. Radioisotopic detection can utilize autoradiographic or scintillation counting methods. A triple capture method using a radioactively-labeled detector probe can achieve a detection limit of 0.2 amol (100fg) of viral nucleic acid (Morrissey et al., 1989b). Nonisotopic labeling of probes with digoxigenin (DIG) (Boehringer-Mannheim) and subsequent detection with Lumi-Phos substrate is advantageous over isotopic labeling of probes in that the probes are stable over a period of months to years and are much safer to personnel. The sensitivity of DIG-labeled

probes is comparable to that of isotope labeled probes (Thompson and Gillespie, 1990).

The polymerase chain reaction (PCR) is a technique that amplifies a specific sequence of target DNA exponentially. Each cycle in the reaction involves denaturing the DNA, annealing the primers, and extending the primers across the templates with DNA polymerase. This technique has revolutionized the detection of RNA viral pathogens, but reverse transcription is required to produce a cDNA of the RNA sequence prior to PCR amplification (Lanciotti et al, 1992). PCR has been used to detect genome segment 7 of BTV in tissue samples, but was unable to detect <1.6 TCID₅₀/ml BTV RNA in blood samples (Wade-Evans et al., 1990). RT-PCR has been used to diagnose infections with prototype U.S. strains of BTV, but problems occurred, including the difficulty of liberating and recovering the viral nucleic acid from infected cells and the presence of poorly defined interfering molecules found in blood and other clinical specimens (Dangler et al., 1990a). To increase the clinical application, PCR and RTC hybridization assays for BTV diagnosis must include rapid techniques that result in both sensitive and specific detection of the virus.

The hypothesis for this study was that RTC hybridization can be a sensitive and specific technique for the detection of BTV. The specific aim of this study was to

develop the RTC hybridization assay to detect BTV in known virus stock cultures and then in BTV-infected *Culicoides*. A revised hypothesis incorporated PCR technology to increase the sensitivity of the RTC hybridization assay. This revised hypothesis stated that the combined RTC-RT-PCR technique can be a sensitive and specific technique for the detection of BTV. Using the combined RTC-RT-PCR technique, viral RNA can be directly amplified from cell-associated virus without the use of extensive (i.e. phenol and chloroform) extraction and purification techniques.

B. Materials and Methods

To optimize the sensitivity and specificity of the RTC hybridization assay, all initial testing was conducted using stock BTV preparations. After standardization of the assay, *Culicoides* pools were prepared containing both BTV-infected and non-BTV-infected *Culicoides*.

1. Viruses

Five prototype strains of BTV that occur in the U.S. (BTV serotypes 2, 10, 11, 13, and 17) and two strains of epizootic hemorrhagic disease of deer virus (EHDV-1 and EHDV-2) were tested in the following studies. These were kindly provided by the USDA/ARS-Arthropod-Borne Animal Diseases Research Laboratory (ABADRL) (Laramie, Wyoming). BTV-2, the Ona B strain, was originally isolated from cattle blood collected in Ona, Florida in 1982. BTV-10 was the BT-

8 strain originally isolated from sheep blood collected in California in 1953. BTV-11 was the BT station strain originally isolated from lamb blood in Texas in 1962. BTV-13 was strain 67-41B originally isolated from cattle blood collected in Idaho in 1967. BTV-17 was strain 62-455 originally isolated from the blood of a sheep in Wyoming in 1962. EHDV-1 was the New Jersey strain originally isolated from a white-tailed deer in 1955. EHDV-2 was the Alberta, Canada strain originally isolated from a white-tailed deer in 1962. One strain of reovirus (Reo-3), Dearing strain, was also tested and was kindly provided by Dr. Dennis Knudson, CSU, Colorado. Passage histories of these virus stocks have been described elsewhere (R. Schoepp, CSU dissertation, 1989).

The viruses were propagated in BHK-21 cells in Leibovitz-15 medium containing 10% fetal bovine serum. The cells were harvested at 3+ CPE, sonicated, and the debris was pelleted. The supernatant was stored at -70°C and thawed when needed for the following assays. Virus titers were determined by plaque assay in BHK-21 cells (R. Schoepp, CSU dissertation, 1989).

2. Positive and negative control *C. variipennis*

Negative control flies used in each test were obtained from the AK colony and kindly provided by the USDA/ARS-ABADRL in Laramie, WY. This colony originated from field

material collected in 1973 from Idaho (Jones and Foster, 1978). BTV-infected adult *Culicoides* were obtained by intrathoracically inoculating adults with each of the 5 U.S. prototype viruses (Jochim and Jones, 1966).

3. Probe preparation

A cDNA of the L3 genome segment of BTV-17 was cloned into pBR322 and was designated plasmid pBTV17-3. This plasmid was kindly provided by Dr. Polly Roy, University of Alabama--Birmingham. Segment L3 codes for viral polypeptide VP3, and is group reactive under low hybridization stringency conditions (Purdy et al., 1984). This probe hybridizes with all five U.S. BTV serotypes and fourteen other BTV serotypes, but does not hybridize to the related EHDV deer.

A transcription plasmid was previously constructed by subcloning a 674 bp fragment of the cDNA from pBTV17-3 into the Bam HI site of pGEM-1 (Schoepp et al., 1990) (Figure 5.1). The construct, named pRSG1.674, yielded RNA probes of positive or negative sense with respect to viral mRNA by transcription from the SP6 or T7 promoter, respectively. The negative sense probe was the detector probe in the RTC assay.

Radioactive detector probes were prepared by transcription of RNA from plasmid pRSG1.674 with bacteriophage T7 RNA polymerase (Promega) and [³²P] UTP

(Amersham) using a Riboprobe labeling kit (Promega) following the manufacturer's instructions. The transcription reaction consisted of 1 ug linearized (HindIII) pRSG1.674 DNA template, 2.5mM of each nucleoside triphosphate (rUTP, rATP, rGTP, and rCTP), 1X transcription buffer (Boehringer-Mannheim), 50uCi alpha-³²P-UTP (Amersham), 1 unit RNasin, 5-15 units of T7 RNA polymerase (Boehringer-Mannheim), 10uM dithiothreitol (DDT) in diethyl pyrocarbonate (DEPC)-treated water. Probe was separated from unincorporated nucleotides using a Sephadex G-50 spin column. The activities of the probes were determined by scintillation counting of trichloroacetic acid precipitates.

Nonradioactive detector probes were prepared by transcription of RNA from plasmid pRSG1.674 with digoxigenin-UTP, using the Genius System RNA labeling kit (Boehringer Mannheim) and T7 RNA polymerase following the manufacturer's instructions. The labeled RNA was precipitated with LiCl (4M) and ethanol, centrifuged, dried under vacuum, and resuspended in 100 ul DEPC-treated water. Labeling of these probes was determined by the addition of ³²P UTP as a tracer in the reaction and subsequent scintillation counting of trichloroacetic acid precipitates.

4. Synthesis of oligodeoxynucleotides

The length of segment L3 is 2772 bp. The detector probe, pRSG1.674 was transcribed from a 674 bp portion of

the L3 gene and is complementary to nucleotides 438 to 1112 on the sense RNA strand. Oligodeoxynucleotide probes complementary to the sense strand of segment L3 and derived from sequences adjacent upstream and downstream to the 674 bp fragment of pRSG1.674 comprised the capture probes (Table 5.1).

Table 5.1. Sequences of BTV synthetic oligodeoxynucleotides

<u>Oligo</u>	<u>Segment</u>	<u>5'Base Position</u>	<u>Sequence (5'-3')</u>
Capture-D	3	1115	ACCTACAACGCCAGCGACCATTCTTACTGCCGG
Capture-U	3	412	CCGCGTATAAACCGCACTTTC
PCR Primer 1	3	485	GGAGGTTGCTGAACCAGAGGTGTT
PCR Primer 2	3	1032	CATTAAGTTGCTGTGCGGTTGGAG
Detector Probe 3	3	438	674bp length

The downstream capture probe sequence was complementary to nucleotides 1115 to 1149 on the sense RNA strand and was located three bases downstream from pRSG1.674. The upstream capture probe sequence was complementary to nucleotides 412 to 433 on the sense strand and was five bases upstream from pRSG1.674. The capture probes have a dissociation temperature of at least 42°C and a GC content of more than 50% (Morrissey and Collins, 1989). The selected capture probe sequences need to be as close as possible to the

detector probe sequence without overlapping to prevent degradation of non-hybridized single-stranded RNA. However, exactly adjacent probes are difficult to select due to the conditions of disassociation temperature and minimum GC content that need to be met. Therefore, capture probes should be within 10 bp of the detector probe for optimum hybridization.

PCR primers were selected from sequences within the detector probe sequences from segment L3 (Table 5.1). The PCR primer 1 sequence was complementary to the sense RNA strand and started at nucleotide 485 while the PCR primer 2 sequence was complementary to the antisense strand and started at nucleotide 1032. Both PCR primers were 24 bp long and had a GC content greater than 50%. In the reverse transcription-polymerase chain reaction assay, these primers amplified a 547 bp piece.

Oligonucleotide PCR primers and capture probes were synthesized on an Applied Biosystems 380B automated DNA synthesizer and purified by HPLC. The concentration of the primers and probes were adjusted to 0.2 ug/ml and aliquots were stored at -20°C.

5. Preparation of poly (dA)-tailed capture probes

Capture probes were poly dA-tailed by incubating a 0.1mM solution of the oligonucleotide overnight at 37°C in 0.1M potassium cacodylate (pH7.0), 1mM dATP, 4mM MgCl₂, 1mM

2-mercaptoethanol, 0.1mg/ml acetylated BSA, and 100 units of terminal deoxynucleotidyl transferase (Pharmacia). Tail lengths averaged 100-300 nucleotides as assessed by electrophoresis through 4% NuSieve (FMC) agarose gels in 1X TAE buffer.

6. Preparation of oligo (dT) magnetic beads

5'-amine-terminated oligo (dT)₁₄ synthesized by standard phosphoramidite chemistry and purified by reverse-phase HPLC were linked to COOH-derivatized ferromagnetic particles (Advanced Magnetics, Cambridge, MA), using 0.1 M EDAC [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide] (Sigma, St. Louis, MO) to activate the COOH groups. The beads were washed with sterile water three times, diluted to 0.1% solids with bead prehybridization buffer [0.1 M Tris-HCl (pH 7.5), 0.01 M EDTA, 4% BSA, 0.5% SDS, and 0.05% bronopol)] and incubated at 68°C for 4 hours. The beads were allowed to cool overnight and the next day were washed 3 times with sterile water, diluted again to 0.1% solids with prehybridization buffer, and stored in 0.02% sodium azide. On the day of use the beads were washed once with prehybridization buffer and resuspended in the same buffer to a concentration of 2 mg/ml.

7. Preparation of poly (dT) nylon

Poly (dT)₄₀₀₀ (Sigma) was diluted to 500 ug/ml in 1.5 M NaCl, 0.5 M MgCl₂, and 0.2 M Tris-HCl (pH 8.0) and 3 ug/slot

was filtered onto Magnagraph nylon membrane (MSI) using a slot-blot apparatus. Following application, the filters were dried by applying 0.2 ml of a 17:1:1:1 mixture of ethanol:methanol:isopropanol:water per slot, and exposed to ultra-violet (254 nm) light at 650 uW/cm² for 3 min. Prior to use in the RTC assay, the membranes were treated by filtering through two 0.2 ml aliquots of 2.5 M GuSCN, 0.2 M Tris HCl (pH 7.5), 40 mM EDTA, 0.1% acetylated BSA, 1 ug/ml denatured salmon sperm DNA, and 0.5% Sarkosyl.

8. Preparation of cell culture analyte

BTV-infected cell medium was were heated to 96°C for 5 min and then cooled immediately on ice to separate the double-stranded RNA before use in the RTC assay.

9. Preparation of *Culicoides* pools

The denaturation solution (900 ul) consisting of 5 M GuSCN, 0.4 M Tris-HCl (pH 7.5), 0.08 M EDTA, and 1.0% Sarkosyl was added to each pool of midges, and the mixture was triturated with a tissuemizer. Fly debris was removed by centrifugation at 14,000 rpm in a microcentrifuge. The supernatant was diluted to 2.5 M GuSCN, and stored until used at -20°C. Before assay, the supernatant was heated to 96°C for 5 min and then cooled immediately on ice.

10. RTC procedure

Initial formation of the ternary complex was accomplished by mixing the sample to be assayed with

approximately 1.0 ng of labeled RNA probe and 50 nmol of the capture probe in 250 μ l of a hybridization medium containing 3.0 M GuSCN, 0.2 M Tris-HCl (pH 7.5), 0.04 M EDTA, 0.5% Sarkosyl, and 10% dextran sulfate-5000. Hybridization was performed at 40°C for 15 min. Following initial hybridization, 500 μ l of the oligo (dT)-derivitized paramagnetic beads (62.5 pmol of oligo dT) (1mg/ml) in bead prehybridization buffer was added to capture the ternary complex--the GuSCN concentration decreased during this step from 3.0M to 1.0M. The mixture was vortexed for 10 sec and the oligo (dT)-poly (A) hybrids were formed by incubating the mixture for 5 min at 40°C in the reaction tube. A magnet was applied to draw the bead-capture probe-target-RNA probe hybridization complex to the side of the reaction tube. The supernatant containing the unannealed RNA probe was decanted and 600 μ l of bead wash solution containing 0.5 M GuSCN, 40 mM Tris HCl (pH 7.5), 8 mM EDTA, 0.5% Sarkosyl, 0.5% acetylated BSA, and 0.5% bronopol (a preservative) was added. The magnet was removed and the beads were then vortexed. The post-hybridization wash was repeated twice more. The magnet was applied and the supernatant was discarded after each wash. Two hundred and fifty μ l of prewarmed (37°C) elution buffer [2.5 M GuSCN, 0.2 M Tris HCl (pH 7.5), 40 mM EDTA, 0.1% acetylated BSA, 1 μ g/ml denatured salmon sperm DNA, and 0.5% Sarkosyl] was added and the

mixture was incubated at 37°C for 5 min, releasing the ternary complex from the beads. A magnet was reapplied and the supernatant was transferred to a new reaction tube containing fresh oligo (dT) derivitized beads. The ternary complex was reattached to the beads by incubating at 40°C for 15 min as described above. The beads were washed three more times as described above. Following the final wash, the ternary complex was eluted from the beads in 250 μ l thermal elution buffer (0.5 M NaCl, 0.1 M Tris-HCl [pH7.5], 0.01 M EDTA, 0.5% Sarkosyl, 1 μ g/ml *E. coli* DNA and 0.1 mg/ml acetylated BSA) for 5 min at 69°C. The nucleic acids were blotted by filtering (0.05 ml/min) through preblocked poly (dT)₄₀₀₀ nylon.

11. Detection of radioactive label

After drying thoroughly, the poly (dT)₄₀₀₀ nylon filters were cut into individual squares, each containing one blotted sample, and placed into separate glass vials. Scintillation fluid was added to each vial and the presence of hybridized radioactive probe was detected in a liquid scintillation counter for each sample. Samples were considered positive if the total counts/min of the sample were greater than the total counts/min of the control.

12. Detection of non-radioactive label

After drying thoroughly, the filters were processed using the Genius detection system (Boehringer Mannheim)

according to manufacturer's instructions. In brief, the filters were equilibrated in buffer A (100 mM Tris-HCl [pH 7.5], 150 mM NaCl) for 5 min and incubated in buffer B (2% [w/v] blocking reagent dissolved in buffer A for 3 hours at 20°C with gentle shaking. The filters were then incubated in a solution of diluted anti-digoxigenin alkaline phosphatase conjugate in buffer B for 30 min at 20°C with gentle shaking. Filters were then washed twice for 15 min each in buffer A and equilibrated in buffer C (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 50 mM MgCl₂) for 2 min. The filters were then transferred to the Lumi-Phos 530 substrate (Boehringer-Mannheim) for 1 min and incubated at 37°C for 30 min in a sealed plastic bag. The filters were exposed to X-ray film for periods from 5 to 15 min. Samples were considered positive if the sample band intensity was greater than that of the control band intensity.

13. RTC-RT-PCR procedure

For the first RTC step, analyte, poly dA-tailed capture probe (1ul), labeled RNA probe and GuSCN solution were combined. The mixture was incubated at 40°C for 20 min, 1 mg of oligo (dT)-labeled magnetic beads were added to form a ternary complex (Figure 5.2), and the mixture was incubated at 40°C for 10 min. After washing the beads three times with the bead wash solution, the beads were resuspended in DEPC-treated water to 1/100 dilution. The

ternary complex was not eluted from the beads but, rather, the ternary complex/bead mixture could then be used in the RT-PCR procedure.

14. Reverse transcription and DNA amplification

One ul of ternary complex/bead mixture was added to 1 ul of the set of PCR primers (final concentration 10 pmol/ul each), 4.5 ul water, and 1 ul methyl mercuric hydroxide (100mM). The mix was incubated for 10 min at room temperature (RT) and 1 ul of 2-mercaptoethanol (350 mM) was added to reduce the methyl mercuric hydroxide. Two ul of the 10X reverse transcriptase buffer [500mM Tris-HCl pH 8.3, 750 mM KCl, and 30 mM MgCl₂] were added to 6 ul of dNTPs (2.5 mM each), 2 ul DTT (100mM), 0.5 ul RNasin (Promega), and 1 ul M-MLV reverse transcriptase (BRL, 200 units) and incubated at 37°C for 1 hour. Eighty ul of the PCR reaction mix containing 8 ul of 10X PCR buffer II (500 mM KCl, 100 mM Tris-HCl pH 8.3) (Perkin-Elmer), 6 ul MgCl₂ (25mM), 0.5 ul *Taq* polymerase (2 units) (Perkin-Elmer), and 65.5 ul water were then added to the reverse transcriptase mix. The mixture was overlaid with 50 ul light mineral oil (USB) and placed in a Perkin-Elmer thermocycler. Cycles were 94°C for 2 min, then 35 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 2 min. After the last cycle the samples were incubated at 72°C for 10 min to complete the primer extensions and then held at 4°C until further analyzed.

15. Analysis of amplified DNA

Aliquots of the amplification products were separated by electrophoresis through 0.5X Tris-borate-EDTA (TBE) 1.4% agarose gels, stained with ethidium bromide, and photographed on a UV transilluminator. Samples were considered positive for BTV when a single band of DNA, 547 bp in size, was evident. A 123 bp ladder was used as a size marker in all gels.

16. Southern hybridization analysis

The agarose gels were soaked in 1.0 M NaCl/0.5 M NaOH twice for 15 minutes each and then soaked in 1.5 M NaCl/0.5 M Tris-HCl, pH 7.4 twice for 15 minutes each. The amplification products were then transferred to a nylon membrane (Magnagraph, MSI) by capillary transfer in 10X SSC (standard saline citrate) (1 X SSC is 0.15M sodium citrate, 1.5 M sodium chloride) overnight. The blots were exposed to a U.V. source (160 J/m^2) for 3 min at a distance of 15 cm to cross-link the DNA to the filter and then the blots were baked at 80° in a vacuum oven for 2 hours to further bind the DNA to the filter. Blots were hybridized immediately or were stored in sealed plastic bags at 4°C until hybridization.

17. Prehybridization/Hybridization of the Southern blots

Membranes were placed in prehybridization buffer that consisted of 6X SSC, 5X Denhardt's solution, 0.5% SDS, 100 ug/ml fragmented, denatured herring sperm DNA, and 50% formamide (pH 7.4) for 2 hours at 42°C in a polyethylene heat-sealed bag in a shaking water bath. Membranes were then placed in fresh prehybridization buffer with the probe added, and incubated overnight in a heat sealed bag in a shaking water bath at 42°C. Membranes were washed twice at 20°C for 5 min each with 2X SSC/0.1% SDS then twice at 42°C for 15 min each with 0.1X SSC/0.1% SDS.

18. Nonradioactive detection using Lumi-Phos

After drying thoroughly, the filters were blocked with the Genius system (Boehringer Mannheim) blocking reagent, incubated with anti-digoxigenin Fab conjugated to alkaline phosphatase, reacted with Lumi-Phos 530 substrate, and exposed to x-ray film, as described previously.

C. Results

1. Defining optimum parameters for the RTC

To define optimum parameters of the RTC assay for the detection of BTV, varying concentrations of analyte, capture probe, guanidinium thiocyanate and degrees of temperature were tested. For optimum capture probe concentration, four reaction mixtures, which were identical except for the

concentration of downstream capture probe, and one control mixture containing no capture probe, were assayed. The concentrations used were 0.5, 1.0, 2.0, and 3.0 pmol of capture probe. A radioactively-labeled detector probe was used and hybridization was performed with one capture. Following elution, the eluant was counted and the results are depicted in Figure 5.3. The upstream capture probe was assayed using the same experimental design, but CPM results were much lower than expected (data not shown) and it was decided to use the downstream capture probe for the following experiments. Hybridization efficiency increased as capture probe concentration increased to an optimal concentration of 2 pmol concentration and then efficiency declined.

To determine the optimum guanidinium thiocyanate concentration in the hybridization buffer, seven hybridization reactions, which were identical except for the GuSCN concentration were prepared, along with two control reactions, including one with no virus and one with no capture probe. The seven concentrations were 1.5, 1.75, 2.0, 2.25, 2.5 2.75, and 3.0 M GuSCN. A radioactively labeled probe was utilized with the downstream capture probe in a hybridization with one capture (Figure 5.4) and two captures (Figure 5.5). Optimum salt concentration in both captures appeared to be 3M.

The optimization of incubation temperature using a radioactively-labeled detector probe, the downstream capture probe, and varying hybridization incubation temperature is shown in Figure 5.6. The temperatures utilized were 31°, 34°, 37°, 40°, and 43°. Forty degrees celsius appeared to be the optimum temperature of hybridization, and was used in all further experiments.

Finally, to determine the optimum concentration of oligo (dT)-linked magnetic beads for capture, six hybridization reactions, which were identical except for the concentrations of magnetic beads, were prepared along with one control reaction containing magnetic beads lacking oligo (dT). The concentrations were 2.0, 1.5, 1.0, 0.5, 0.25, and 0.1 mg/ml. The optimum concentration was 1 mg/ml beads per reaction, and was used in all further experiments (data not shown).

The limit of detection of BTV in the assay is shown in Figure 5.7, which represents one capture hybridization using a radioactively-labeled detector probe and the downstream capture probe. The titer of the virus, BTV-17 was $10^{4.1}$ TCID₅₀/ml. Virus titers of 62.5 TCID₅₀ yielded detectable signal.

2. Radioactively-labeled probes vs. non-radioactively-labeled probes

Radioactively-labeled probes were compared to non-radioactive-labeled probes to determine the optimum label to use in developing this assay. Two identical slot-blot filters containing viral RNA extracted from BTV-infected BHK-21 cells are shown in Figure 5.8A,B. Lanes A1-A3 on both filters contained RNA extracted from BTV-17-infected BHK-21 cells. Lane A1 contained 0.1 ug/ml RNA, lane A2 contained 10 ng/ml RNA, and lane A3 contained 1 ng/ml RNA. Lanes B1-B3 on both filters contained RNA extracted from BTV-10-infected BHK-21 cells. Lane B1 contained 0.1 ug/ml RNA, lane B2 contained 10 ng/ml RNA, and lane B3 contained 1 ng/ml RNA. Lane C1 contained no sample. Lane C2-C3 contained RNA extracted from BTV-13-infected BHK-21 cells. Lane C2 contained 10 ng/ml RNA and lane C3 contained 1 ng/ml RNA. Negative control RNA extracted from uninfected BHK-21 cells (0.1 ug/ml) was blotted in lanes A4, B4, and C4. Figure 5.8A shows the blot hybridized with ^{32}P -labeled probe, while figure 5.8B shows the blot hybridized with digoxigenin-labeled probe. The non-radioactively-labeled probe detected by chemiluminescence (Figure 5.8B) was more sensitive (higher intensity of bands, visible even at the 0.1 ng/ml level) and provided results more quickly than the radioactively-labeled probe (Figure 5.8A) (5 min versus 48

hours). The non-radioactively-labeled probe was used from this point on in the assay.

3. Chemiluminescent detection adaptation of the RTC assay

Figure 5.9 shows the chemiluminescent detection of BTV using a digoxigenin-labeled detector probe using titers described in Figure 5.7. The detection limit appeared to be the same as that seen with the radioactively-labeled probe.

The RTC assay using a digoxigenin-labeled probe also detected viral RNA in BHK cells and *C. variipennis* infected with each of the five serotypes after one capture (Figure 5.10). BTV-infected BHK cells and *C. variipennis* assayed without capture probe yielded negative results. However, low signal was detected in the BTV-infected adult flies.

4. RTC-PCR on BTV-infected cell culture

To increase sensitivity, the RTC was then combined with RT-PCR. The RTC-RT-PCR assay detected all five BTV serotypes (Figure 5.11) in infected BHK cell cultures. EHDV-1, EHDV-2, reovirus type 3, and controls yielded negative results. Southern analyses (Figure 5.12) yielded hybridization signals with all five serotypes, but negative results for EHDV-1, reovirus type 3 and controls. EHDV-2 yielded a slight signal only detectable by Southern blot hybridization.

5. RTC-PCR on BTV-infected *C. variipennis*

The RTC-RT-PCR also detected all five US BTV serotypes in infected *C. variipennis*; negative results were obtained with uninfected *C. variipennis* (Figure 5.13). Southern blot analyses (Figure 5.14) yielded hybridization signals for all five U.S. serotype-infected *C. variipennis* and no signal with uninfected *C. variipennis*.

6. RTC-PCR--Effect of analyte dilution

The importance of analyte dilution before reverse transcription and amplification is demonstrated in Figure 5.15. Analyte-bead suspensions diluted only 1:10 before amplification (lanes 3-6) were not detectable by either agarose gel electrophoresis or Southern analyses, while analyte-bead suspensions diluted 1:100 (lanes 7-12) yielded easily detectable results with both agarose gel detection and Southern blot analysis (Figure 5.16).

7. RTC-PCR--Investigation of non-specific binding

Theoretically, the BTV analyte could nonspecifically bind to the beads and be subsequently amplified by PCR, instead of specifically being captured by the RTC and amplified. To investigate this possibility all samples in Figure 5.15 were amplified not only with the segment 3 primers but also with segment 2 and 6 primers (described in Chapter IV). No extra bands were amplified in the samples except for the positive control, which contained all 10

segments of the virus (lane 2). Therefore, only segment 3 was present in samples first processed by the RTC assay, which removed all of the other BTV segments.

D. Discussion

A novel molecular-based assay that combines the RT-PCR with the RTC hybridization methodology for the detection of BTV analyte has been developed. After defining the optimum parameters for the RTC assay, the test was enhanced by the incorporation of the RT-PCR assay for greater sensitivity. The assay was also adapted to a nonradioactive format using digoxigenin-labeled probes. The technique was able to detect all five US prototype viruses in both BTV-infected cell culture samples and BTV-infected *C. variipennis* adults. EHDV-2-infected cell culture yielded a slight hybridization signal after a Southern blot hybridization was performed. This signal was not seen when EHDV-2-infected *C. variipennis* were tested. The signal could have been due to a slight cross-reactivity of the probe to EHDV-2, which has been reported to have more homology with BTV than EHDV-1.

Analyte dilution had an important effect on the amplification step of the assay. Analyte-bead suspensions diluted only 1:10 before amplification, produced no amplified product, while analyte-bead suspensions diluted 1:100 did produce amplified product. Thus sample dilution needs to be considered when using higher concentrations of

analyte RNA. Non-specific binding of other BTV gene segments (segments 2 and 6) did not occur in the RTC-RT-PCR assay. No extra bands were amplified in the samples except for the positive control, which contained all of the BTV gene segments.

The RTC-RT-PCR technology looks promising as a diagnostic tool, especially for the detection of BTV in clinical samples such as blood and tissue that contain PCR inhibitory factors. This technique bypasses the need for extensive RNA extraction procedures involving the use of phenol, chloroform and proteinase K extraction steps. Problems associated with RNase degradation are also eliminated because the guanidinium thiocyanate inactivates any RNase that may be present. Future studies should include comparison with other more standardized techniques such as blot hybridization and viral isolation to determine the relative specificity and sensitivity of the assay.

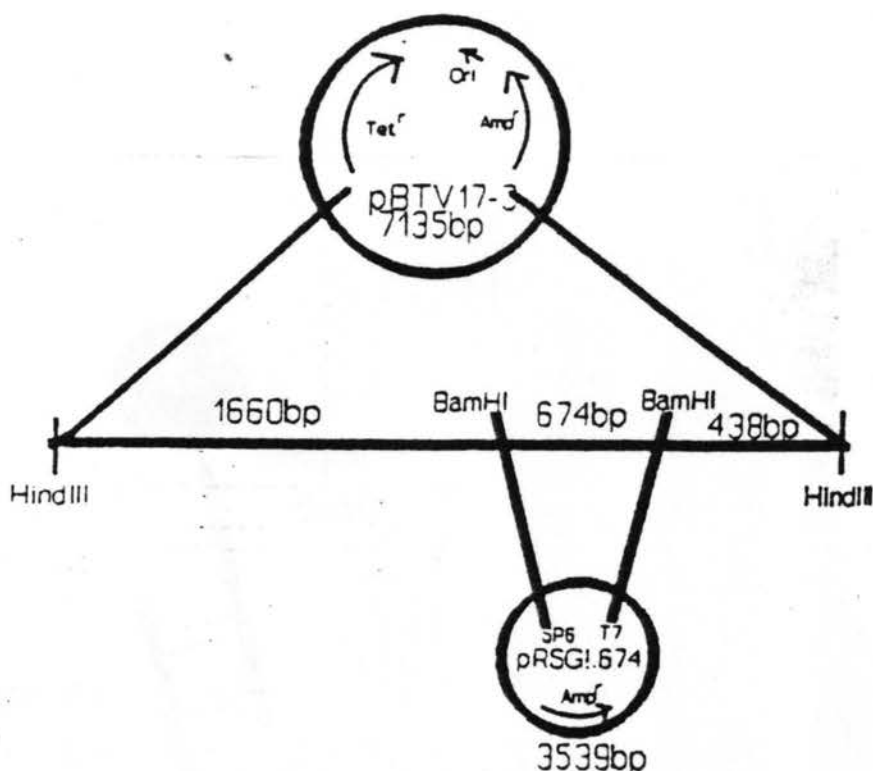


Figure 5.1. Probe constructs pBTV 17-3 and pRSG1.674. cDNA generated from the L3 segment of BTV-17 was cloned into pBR322 and kindly provided by Dr. Polly Roy, University of Alabama. A recombinant in vitro transcription plasmid was constructed by subcloning a 674bp fragment into the Hind III and Bam HI sites of pGEM-1 (Schoepp et al 1990). The construct pRSG1.674 yields RNA antisense probes by transcription from the T7 promoter.

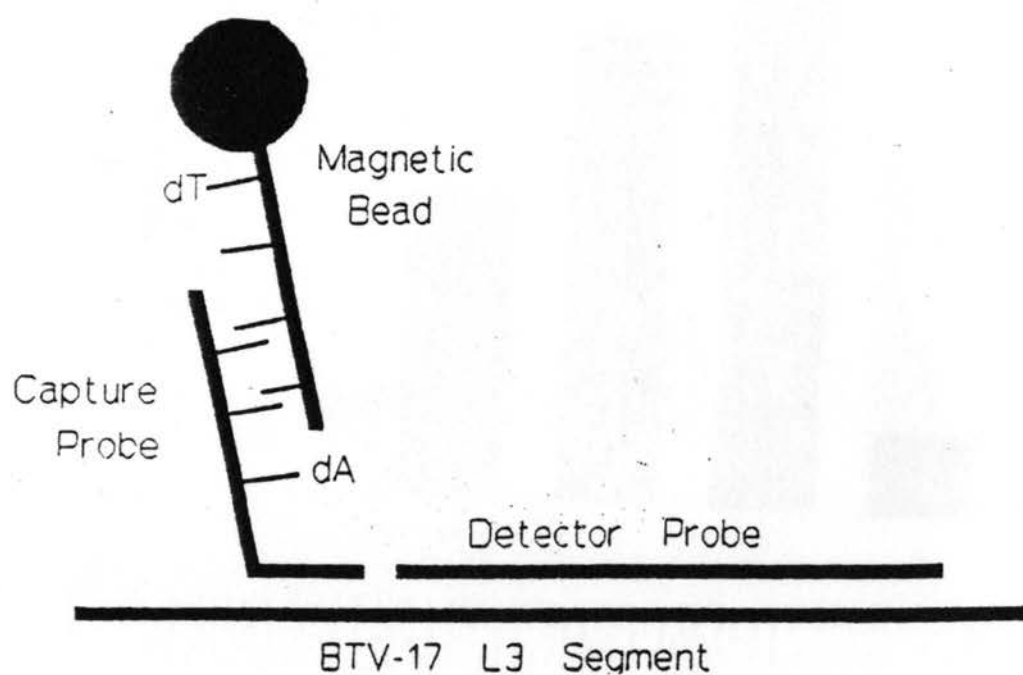


Figure 5.2. Schematic diagram of the RTC hybridization assay. The ternary complex of analyte, capture probe, and detector probe binding to the paramagnetic beads through poly A--oligo T bonds is depicted.

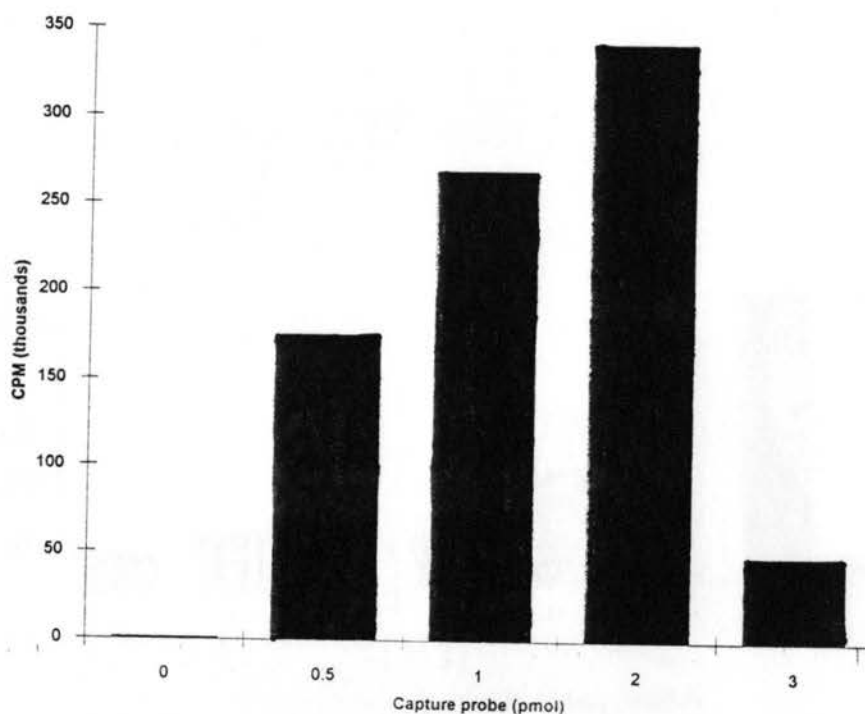


Figure 5.3. Determination of optimum capture probe concentration. To define optimum capture probe concentrations as a parameter of RTC, varying concentrations of capture probe (pmol) were used. Counts per minute (CPM) were determined by scintillation counting of the hybridized radioactively labeled probe after one capture.

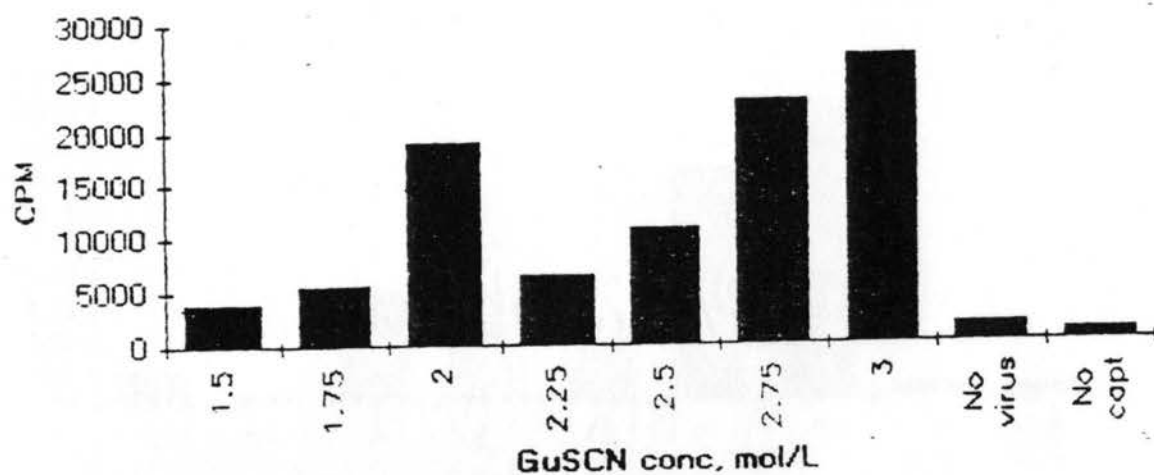


Figure 5.4. Determination of optimum guanidinium thiocyanate concentration (1 capture method), using a radioactively labeled probe.

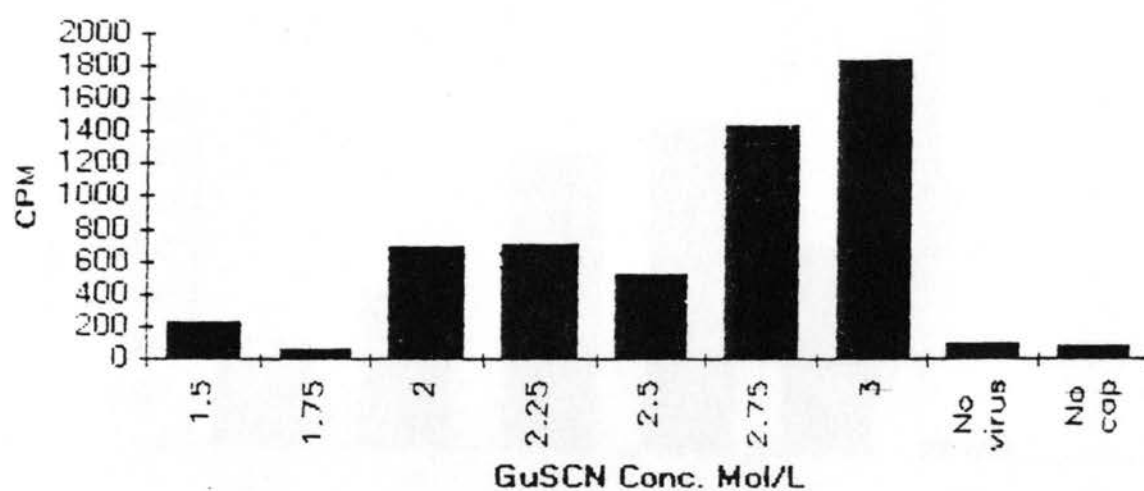


Figure 5.5. Determination of optimum guanidinium thiocyanate concentration (2 capture method), using a radioactively labeled probe.

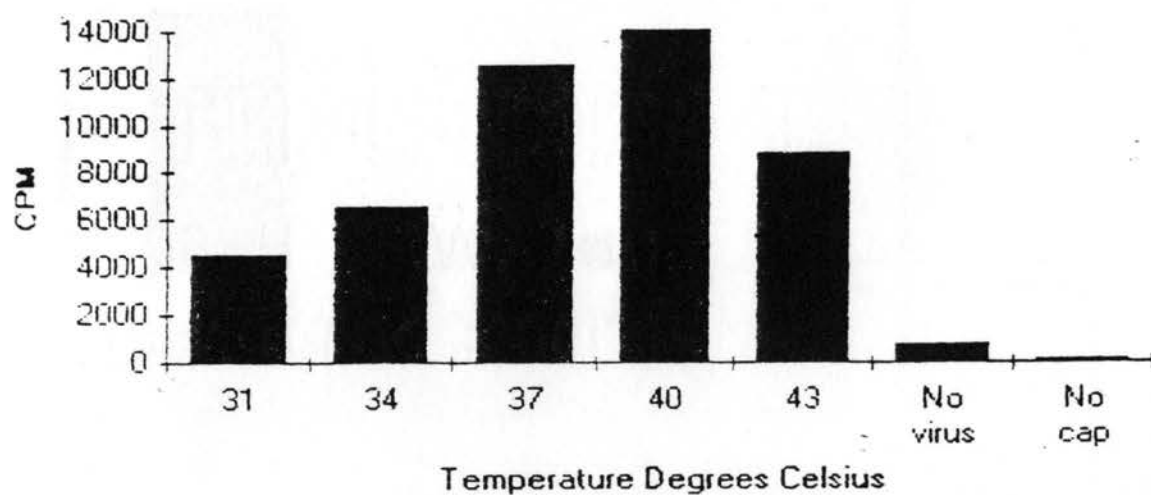


Figure 5.6. Determination of optimum incubation temperature after one capture using a radioactively labeled detector probe.

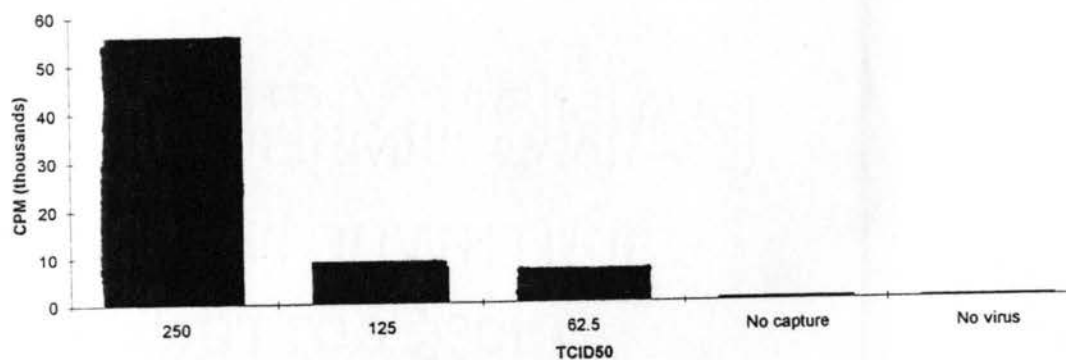


Figure 5.7. Limit of detection for BTV-17 in RTC assay using a ^{32}P -labeled probe. Decreasing amounts of BTV-17 (titer = $10^{4.1}$ TCID₅₀/ml) were assayed with a radioactively-labeled detector probe after one capture. Controls included no capture probe and no virus.

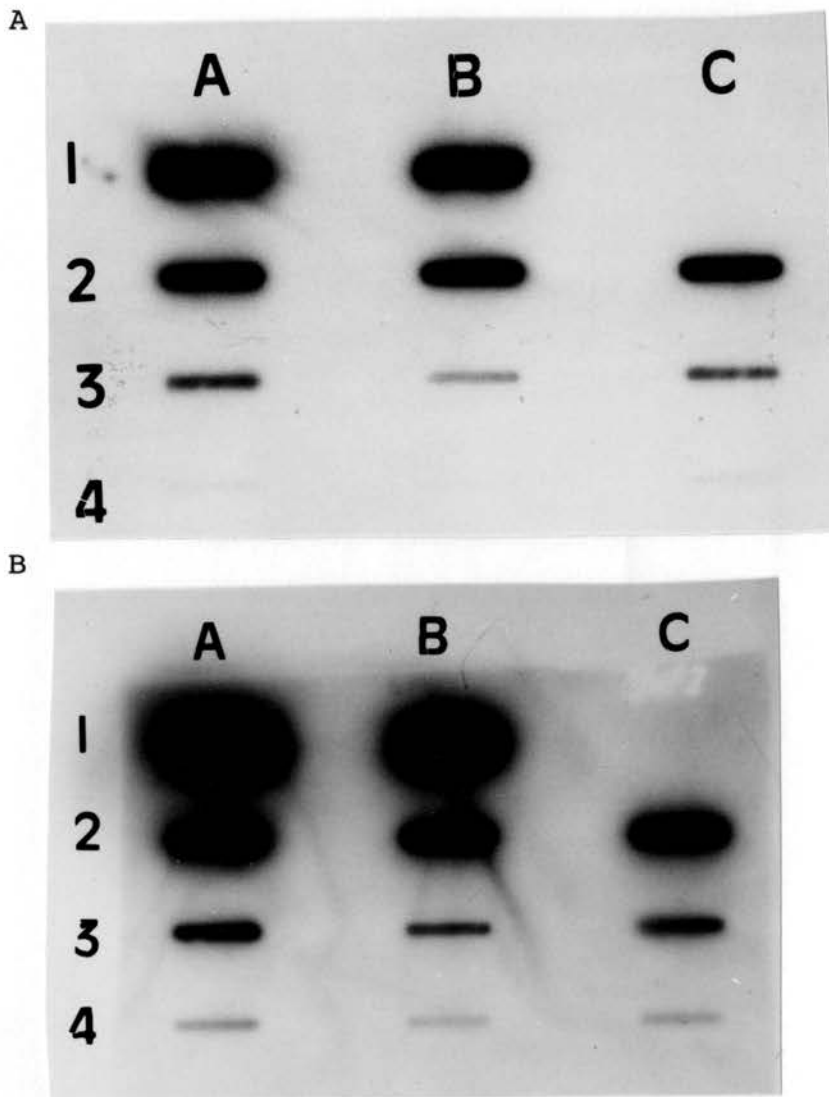


Figure 5.8 A,B. Radioactively-labeled probes compared to non-radioactively-labeled probes. Slot-blots containing BTV RNA extracted from BTV-infected BHK-21 cells were hybridized with ^{32}P -labeled probe (A) or with digoxigenin-labeled probe (B). Lanes A1-A3 contain BTV-17 RNA in decreasing dilutions including 0.1 ug/ml (A1), 10 ng/ml (A2) and 1 ng/ml (A3). Lanes B1-B3 contain BTV-10 RNA in the same decreasing dilutions while lanes C2-C3 contain BTV-13 RNA in dilutions 10 ng/ml (C2) and 1 ng/ml (C3) only. Negative control RNA was blotted in lanes A4, B4, and C4.

TCID₅₀

250
125
62.5
31
No C
No V

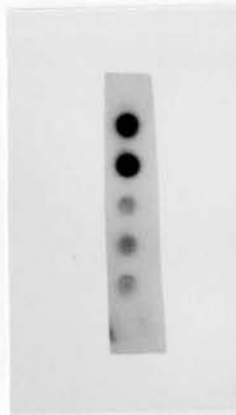


Figure 5.9. Limit of detection of RTC assay for BTV-17 using digoxigenin-labeled probe. Decreasing amounts of BTV-17 (titer= $10^{4.1}$ TCID₅₀/ml) were assayed with a nonradioactively-labeled probe after one capture. Controls include "C" which contains only virus and capture probe, and no detector probe, and "V" which contains only virus, and no capture or detector probe.

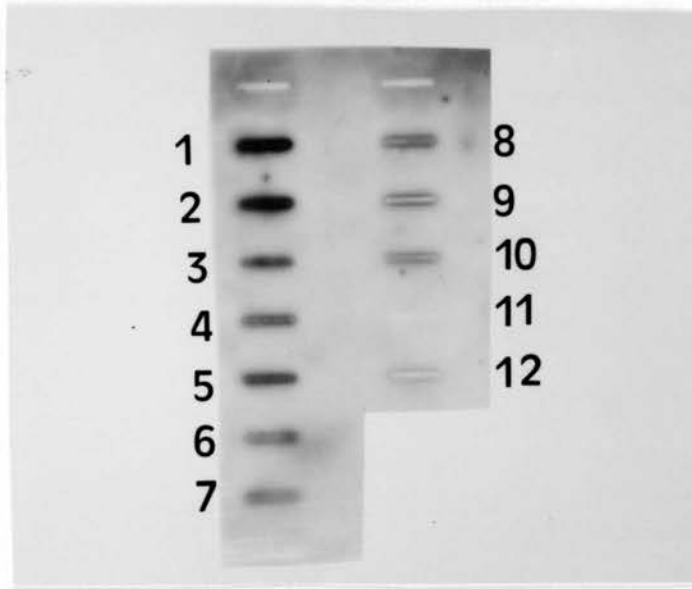


Figure 5.10. RTC detection of BTV-infected cell culture and BTV-infected adult flies. Slot blot of RTC assay using a digoxigenin-labeled detector probe detecting all five prototype US strains of BTV in infected BHK cells and intrathoracically inoculated *Culicoides variipennis* adults. Slots 1-5 are from infected BHK-21 cells; sample 1 is BTV-2, sample 2 is BTV-10, sample 3 is BTV-11, sample 4 is BTV-13, and sample 5 is BTV-17. Samples 6-10 are from BTV intrathoracically inoculated adults; sample 6 is BTV-2, sample 7 is BTV-10, sample 8 is BTV-11, sample 9 is BTV-13, and sample 10 is BTV-17. Slot 11 is from BTV negative BHK cells and slot 12 is from BTV-negative adult flies.



Figure 5.11. Electrophoretic separation of RTC-PCR products of BTV-infected cell culture. Electrophoretic separation (agarose gel, 1.4%) of PCR products from RTC-RT-PCR on five US prototype strains of bluetongue serotypes, two strains of epizootic hemorrhagic disease virus of deer, and one strain of Reovirus. Lane 1--123bp ladder molecular weight marker, Lane 2--BTV-2, Lane 3--BTV-10, Lane 4--BTV-11, Lane 5--BTV-13, Lane 6--BTV-17, Lane 7--EHDV-1, Lane 8--EHDV-2, Lane 9--Reo-3, Lane 10--negative control.

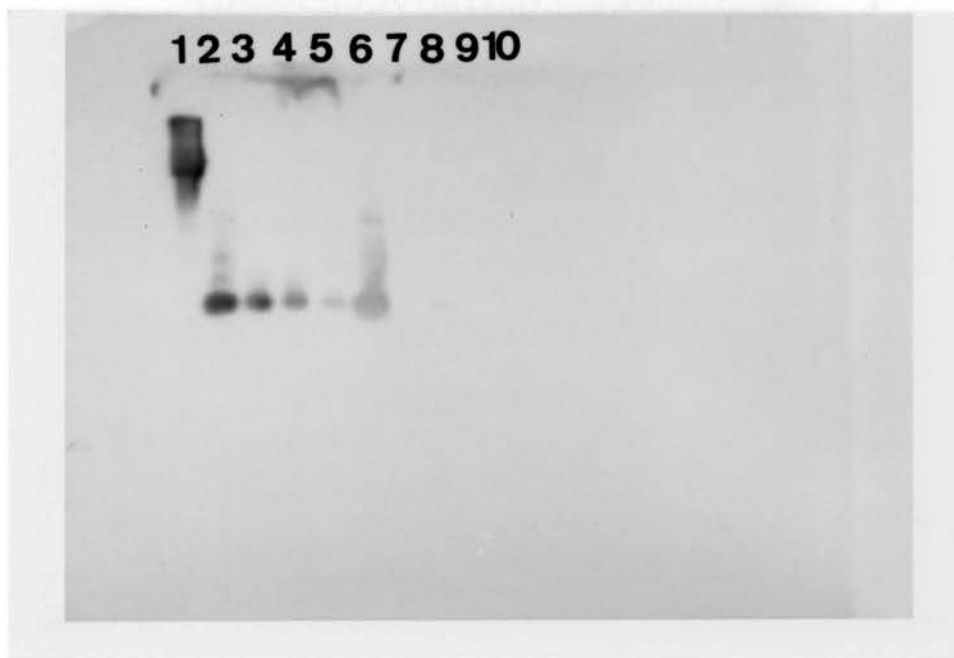


Figure 5.12. Southern blot hybridization of RTC-PCR products of BTV-infected cell culture. Southern blot hybridization of PCR products from RTC-RT-PCR (Figure 3.12) using a digoxigenin-labeled pRSG1.674 probe and exposed to x-ray film for 10 min. Lane 1--123bp ladder molecular weight marker, Lane 2--BTV-2, Lane 3--BTV-10, Lane 4--BTV-11, Lane 5--BTV-13, Lane 6--BTV-17, Lane 7--EHDV-1, Lane 8--EHDV-2, Lane 9--Reo-3, Lane 10--negative control.

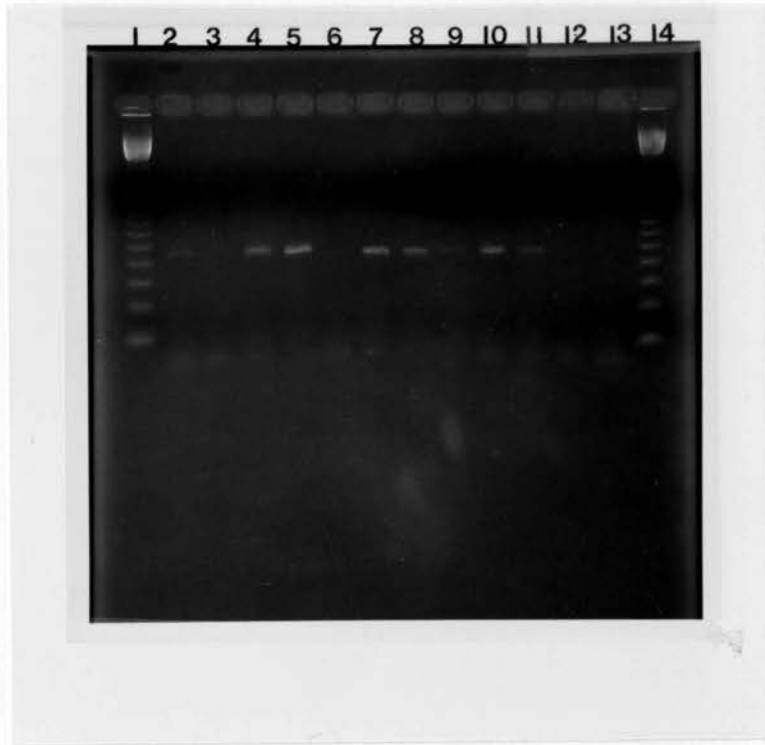


Figure 5.13. Electrophoretic separation of RTC-PCR products of BTV-infected *Culicoides variipennis*. Electrophoretic separation (agarose gel, 1.4%) of PCR products from RTC-RT-PCR on BTV-infected *Culicoides variipennis* adults intrathoracically inoculated with all five prototype US strains of BTV. Lane 1--123bp molecular weight marker, Lane 2--BTV-2 (1 adult), Lane 3--BTV-2 (2 adults), Lane 4--BTV-10 (1 adult), Lane 5--BTV-10 (2 adults), Lane 6--BTV-11 (1 adult), Lane 7--BTV-11 (2 adults), Lane 8--BTV-13 (1 adult), Lane 9--BTV-13 (2 adults), Lane 10--BTV-17 (1 adult), Lane 11--BTV-17 (2 adults), Lane 12--1 uninfected adult, Lane 13--2 uninfected adults, Lane 14--123bp ladder molecular weight marker.



Figure 5.14. Southern blot hybridization of RTC-PCR products of BTV-infected *Culicoides variipennis*. Southern blot hybridization of PCR products from RTC-RT-PCR (figure 3.14) using a digoxigenin-labeled pRSG1.674 probe and exposed to x-ray film for 10 min. Lane 1--123bp molecular weight marker, Lane 2--BTV-2 (1 adult), Lane 3--BTV-2 (2 adults), Lane 4--BTV-10 (1 adult), Lane 5--BTV-10 (2 adults), Lane 6--BTV-11 (1 adult), Lane 7--BTV-11 (2 adults), Lane 8--BTV-13 (1 adult), Lane 9--BTV-13 (2 adults), Lane 10--BTV-17 (1 adult), Lane 11--BTV-17 (2 adults), Lane 12--1 uninfected adult, Lane 13--2 uninfected adults, Lane 14--123bp ladder molecular weight marker.

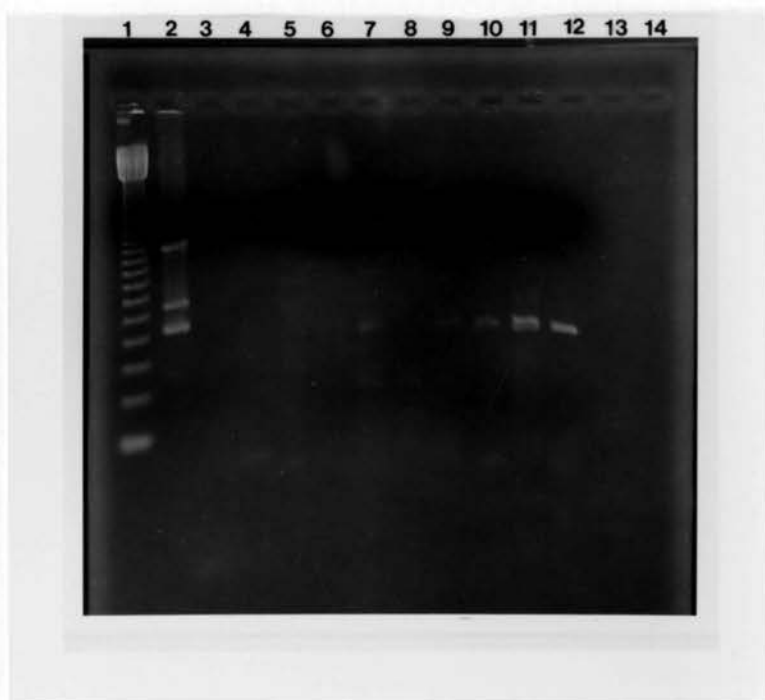


Figure 5.15. Electrophoretic separation of RTC-PCR products visualizing the effects of analyte dilution and non-specific binding. Electrophoretic separation (agarose gel, 1.4%) of PCR products from RTC-RT-PCR of dilutions of virus-attached beads. Lane 1--123bp ladder molecular weight marker, Lane 2--positive control from BTV-17 infected BHK cell culture, Lanes 3 and 4--PCR product derived from BTV-10-infected cell culture that was not passed through the RTC steps of the assay and diluted 1/10, Lanes 5 and 6--RTC-PCR products from BTV-17-infected cell culture diluted 1/10, Lanes 7 and 8--RTC-PCR products from BTV-10 infected cell culture diluted 1/100, Lanes 9 and 10--RTC-PCR products from BTV-13 infected cell culture diluted 1/100, Lanes 11 and 12--RTC-PCR products from BTV-17 infected cell culture diluted 1/100, and Lanes 13 and 14--RTC-PCR products from uninfected cell culture.

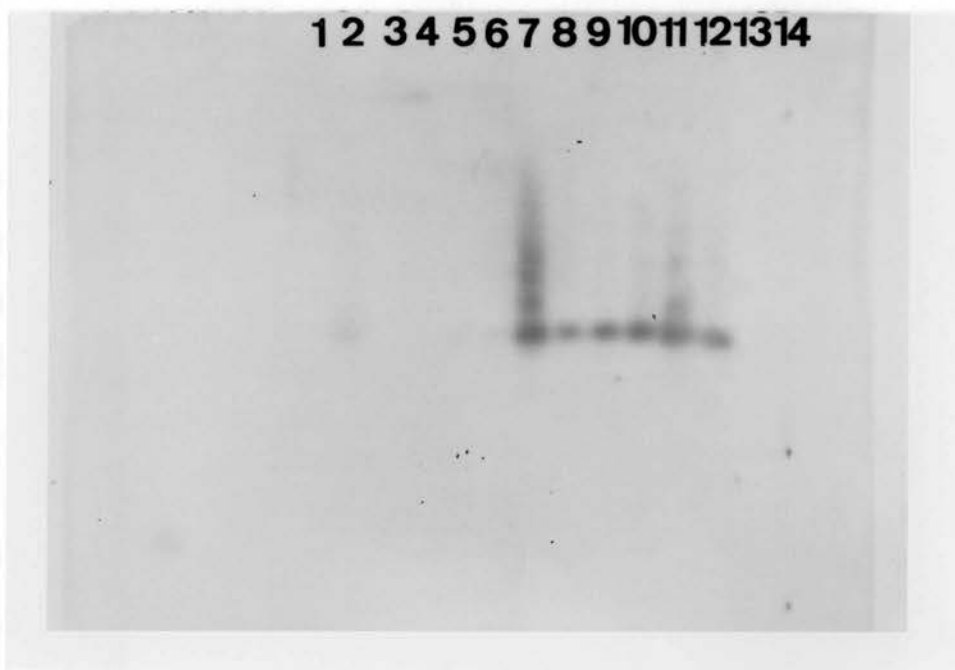


Figure 5.16. Southern blot hybridization of RTC-PCR products visualizing the effects of analyte dilution and non-specific binding. Southern blot hybridization of PCR products from RTC-RT-PCR (Figure 3.16) using a digoxigenin-labeled pRSG1.674 probe and exposed to x-ray film for 10 min. Lane 1--123bp ladder molecular weight marker, Lane 2--positive control from BTV-17 infected BHK cell culture, Lanes 3 and 4--PCR product derived from BTV-10 infected cell culture that was not passed through the RTC steps of the assay and diluted 1/10, Lanes 5 and 6--RTC-PCR products from BTV-17-infected cell culture diluted 1/10, Lanes 7 and 8--RTC-PCR products from BTV-10 infected cell culture diluted 1/100, Lanes 9 and 10--RTC-PCR products from BTV-13 infected cell culture diluted 1/100, Lanes 11 and 12--RTC-PCR products from BTV-17 infected cell culture diluted 1/100, and Lanes 13 and 14--RTC-PCR products from uninfected BHK cell culture.

VI. SUMMARY

BTV remains endemic in many areas of the world, including Colorado, resulting in significant direct and indirect economic losses. Molecular techniques have proven to be efficacious in diagnosing BTV rapidly. Rapid techniques such as RNA hybridization and the polymerase chain reaction can be broadly applied to investigating transmission, overwintering, and other epidemiologic questions, to diagnostic applications concerning economic trade restrictions, and to understanding of the molecular biology of vector and vertebrate host pathogenesis.

An ongoing prospective study assessing the diagnostic efficacy of these molecular techniques to investigate BTV overwintering was conducted in a bonafide field situation in Colorado. BTV activity has been documented in these endemic study areas for the past ten years. During the study period BTV seroconversion occurred in at least one of the sentinel calves. Over 62,000 larvae were collected from the field and tested using standard virus isolation and newer molecular techniques such as RNA hybridization and the capture-antigen ELISA.

Eighty-seven larval pools tested positive by one assay test; 11 pools tested positive by two assay tests. The test concordance for positive larval pools was low. Infectious virus may be present in the larvae in levels lower than the detection limits of the assays or virus may be present in a transcriptionally latent state. In either case, when the larvae are stimulated by morphogenesis into pupae or adults, or there is a change in environmental conditions, such as temperature, BTV may then enter a replicating, infectious virus state. Virus isolation was not a sensitive technique for detecting BTV in these tests, while the ELISA proved to be a satisfactory screening test, especially for the large number of pools studied.

There were significant differences between the larval MFIR's at the Ausman and Fields farm sites in both 1991 and 1992. The larval MFIR at the Ausman farm was double that found at the Fields farm. Significant differences were also found between the diagnostic test MFIR's ($p < 0.001$).

BTV transmission was occurring at the study farm sites in the summer of 1992. One sentinel calf at the Howard farm (next to the Ausman farm) seroconverted; antibodies to BTV were detected in October by cELISA and the confirmatory AGID. The sentinel calf at the Ausman farm appeared to seroconvert; antibodies to BTV were detected in September but this was not confirmed by the AGID. This result could

have been due to the decreased sensitivity seen with the AGID. Six herd calves seroconverted during the summer; four at the Fields farm, one at the Ausman farm and one at the Howard farm. This is an interesting observation since more BTV-infected larvae and adult flies were captured from the Ausman farm than the Fields farm. However, there were fewer calves tested for seroconversion at the Ausman farm.

A proposed mechanism for overwintering is described based on a time-course analysis of the events observed in 1992. BTV-positive larvae peaked in June, BTV-positive adult *C. variipennis* peaked in July, and sentinel and herd animals seroconverted in September and October. These observations would fit the vertical transmission hypothesis time-wise.

These studies did not prove that vertical transmission is the overwintering mechanism of BTV in the study areas since infectious virus was not isolated. However, the studies did provide useful data as to where future efforts should focus. Larvae could be collected and reared to the adult stage, identified to species, and then screened with the ELISA.

To determine host preference and feeding activity, 11,466 insects were collected utilizing drop traps baited with vertebrate animals. The heaviest insect attack rate occurred at the Fields site in September and at the Ausman

site in July. The calf consistently attracted greater numbers of insects than the sheep except for *C. crepuscularis*, which preferred sheep to cattle. Significant differences ($p < 0.001$) were observed in the MFIR's of *C. variipennis* between the Ausman (11%) and Fields (4.5%) farm sites. BTV was also isolated from *Culicoides* such as *C. crepuscularis* and *C. haematopodus* and these species need to be investigated further.

A revised hypothesis could state that BTV may overwinter in temperate regions such as Colorado through vertical transmission of the virus in other *Culicoides* species. *C. variipennis* may act as the epidemic vector, while another species of *Culicoides* act as the endemic and overwintering vector.

The molecular techniques developed in these studies may be most useful in the investigation of BTV epidemiology and transeasonality. The tRNA-PCR assay appears to be a promising tool to identify *Culicoides* species in adult, pupal or larval stages. Mixed or heterogeneous pools of *Culicoides* species could be differentiated and not confused with homogeneous pools of one *Culicoides* species. Preliminary results show approximately 50% of previously unidentified field larvae could be identified as *C. variipennis* by this assay. This test may be useful for identification of other insects economically or medically

important that could be identified at the larval stage so that directed vector control could be instituted.

The RTC-RT-PCR may be useful in the identification of organisms like BTV that are cell-associated and difficult to detect utilizing PCR alone. The RTC assay was combined with RT-PCR technology to increase the sensitivity of the assay. The RTC-RT-PCR could detect all five US prototype viruses in both BTV-infected cell culture samples and BTV-infected *C. variipennis* adults. One advantage of the assay is that the need for extensive RNA extraction procedures is bypassed. Also, problems associated with RNase degradation are eliminated because the guanidinium thiocyanate inactivates any RNase that may be present. Future applications of the RTC-RT-PCR assay may include testing of BTV-infected blood samples that are notorious for negative PCR results because of blood component interference.

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APPENDIX A

Summary of larval pools positive by at least one test

Acc #	Site	Date	Pool	PCR	ELISA	FA	RNA
11020	Fields	4/23/91	E		+	-	-
11247	Fields	6/20/91	D		+	-	-
11404	Aus	8/30/91	G		-	-	+L
11446	Fields	5/8/91	BB		+	-	-
11449	Fields	5/8/91	O		+	-	-
11465	Fields	4/91	101	+	-	-	-
11468	Fields	4/91	95		+	-	-
11470	Fields	4/91	98		+	-	-
11478	Aus	8/30/91	C3	-	-	-	+B/+C
11481	Aus	8/23/91	D4		+	-	-
11484	Aus	8/23/91	C		+	-	-
11497	Aus	8/23/91	T		+	-	-
11583	Aus	8/23/91	I		+	-	-
11620	Aus	8/23/91	W	+	-	-	-
11625	Aus	9/20/91	I	+	-	-	-
11642	Aus	8/23/91	T	+	**+	-	-
11651	Aus	8/23/91	Z		+	-	-
11652	Aus	8/23/91	R	+	-	-	-
11668	Aus	8/23/91	I	+	*+	-	-
11677	Aus	8/30/91	X		-	-	+B
11714	Fields	3/26/92	B		+	-	-
11733	RB	5/19/92	RI		-	-	+V
11735	RB	5/19/92	RN		-	-	+B/+C
11754	Aus	2/28/92	J	+	+	-	-
11756	Aus	2/28/92	G		+	-	-
11762	Aus	3/26/92	W		+	-	-
11767	Aus	3/29/92	T		+	-	+B
11768	Aus	3/26/92	P		-	-	+B
11771	Aus	3/26/92	V		+	-	-
11775	Aus	3/26/92	W		+	-	-
11792	RB	5/19/92	RK		-	-	+C
11795	RB	5/19/92	RH		-	-	+C
11797	RB	5/19/92	RO		-	-	+C
11825	Aus	2/91	44		-	+B	-
11846	Aus	2/91	46		-	+B	-
11902	Aus	6/5/91	X		-	-	+C
11911	Aus	6/5/91	W		-	-	+C

Acc #	Site	Date	Pool	PCR	ELISA	FA	RNA
11917	Aus	6/5/91	W		+	-	-
11929	Aus	6/5/91	W		-	-	+L/+C
11946	Aus	5/7/92	U4		+	-	-
11959	Aus	5/7/92	T3		+	-	-
11980	Aus	5/7/92	L		-	-	+L
12009	Fields	4/23/92	I		+	-	-
12010	Fields	4/23/92	K		+	-	-
12032	Fields	4/23/92	B		+	-	-
12033	Fields	4/23/92	K		+	-	-
12034	Aus	5/7/92	K		+	-	-
13000	Aus	6/26/92	H		-	-	+C/+V
13002	RB	7/10/92	RH		-	-	+C
13026	Aus	6/26/92	K		+	-	-
13033	Aus	6/26/92	H		+	-	-
13037	Fields	7/31/92	E		-	-	+V
13071	Fields	2/3/92	Z		+	-	-
13092	Aus	6/5/92	T		+	-	-
13093	Aus	6/5/92	H		+	-	-
13094	Aus	6/5/92	I		+	-	-
13109	Aus	6/5/92	H		+	-	-
13129	Aus	6/5/92	T		+	-	-
13201	RB	5/19/92	RO		+	-	-
13210	Aus	8/26/92	V		-	-	+L/+B
13258	Fields	6/5/91	BB		+	-	-
13268	Fields	6/26/92	I		-	-	+L
13277	Fields	5/16/92	O		+	-	-
13282	Fields	5/16/92	O		+	-	-
13287	Fields	6/5/91	D		+	-	-
13291	Aus	5/21/91	V		+	-	-
13292	Aus	5/21/91	X		+	-	-
13296	Fields	5/21/91	K		+	-	-
13301	Aus	6/5/91	D		+	-	-
13306	Fields	6/5/91	D		+	-	-
13309	Fields	6/5/91	N		+	-	-
13331	Aus	7/10/92	E		+	-	-
13339	Aus	6/26/92	H		+	-	-
13347	Aus	6/26/92	S		+	-	-
13352	Aus	7/17/92	U		+	-	-
13356	RB	8/14/92	RK		**+	-	-
13417	Aus	8/14/92	L		+	-	-
13418	Aus	9/7/92	H		+	-	-
13449	Aus	6/26/92	H		+	-	-
13453	Aus	6/26/92	I		+	-	-
13500	Aus	6/26/92	X		+	-	-
13507	Aus	5/7/92	L		+	-	-
13541	RB	6/12/92	RN		+	-	-
13553	RB	6/12/92	RN		**+	-	-
13572	Fields	7/10/92	E		+	-	-

<u>Acc #</u>	<u>Site</u>	<u>Date</u>	<u>Pool</u>	<u>PCR</u>	<u>ELISA</u>	<u>FA</u>	<u>RNA</u>
2049	Fields	7/10/92	E		** +	-	-
2067	Aus	7/17/92	T		+	-	-

Acc # = Accession number of pool

See Table 2.1 for pool site designations—Numbered collected in 1991 did not receive a pool site letter

Aus = Ausman site

Fields = Fields site

RB = Red Barn (Howard site)

L = Positive RNA hybridization of larval pool

B = Positive RNA hybridization of BHK-21 passed pool

C = Positive RNA hybridization of C6/36 passed pool

V = Positive RNA hybridization of Vero passed pool

PCR = Polymerase chain reaction

IFA = Indirect fluorescent antibody assay

RNA = RNA hybridization assay

ELISA = Enzyme-linked immunosorbent assay

* = First ELISA was negative; repeat was positive

** = Repeat ELISA was also positive

APPENDIX B

Characterization of pools of insects and corresponding ELISA values

Acc #	Site/Cycle	Date	# and Species	Sample	ELISA Values	
					Neg Con + 3 SD	Pos Con
51000	F-S-1	6/29/92	1 C var	0.1185	0.1724	0.3307
51001	F-C-1	6/29/92	5 C var BF	0.1127	0.1724	0.3307
51002	F-C-1	6/29/92	10 C var NBF	0.1072	0.1724	0.3307
51003	F-C-1	6/29/92	8 C other	0.042	0.09	0.216
51003C	F-C-1	6/29/92	1 C var	0.358	0.409	1.489
51003D	F-C-1	6/29/92	1 C haem	0.401	0.489	2.096
51004	F-C-1	6/29/92	Mosq	0.0982	0.1724	0.3307
51005	F-S-2	6/29/92	1 C var	0.0837	0.1724	0.3307
51006	F-S-2	6/29/92	6 C other	0.323	0.393	1.335
51006B	F-S-2	6/29/92	2 C crep	0.301	0.393	1.335
51006D	F-S-2	6/29/92	1 C haem	0.298	0.393	1.335
51007	F-S-2	6/29/92	Mosq	0.1005	0.1724	0.3307
51008	F-C-2	6/29/92	8 C var NBF	0.1045	0.1724	0.3307
51009	F-C-2	6/29/92	5 C other	0.328	0.409	1.489
51009B	F-C-2	6/29/92	1 C crep	0.415	0.489	2.096
51009F	F-C-2	6/29/92	2 2-spot	0.327	0.409	1.489
51010	F-C-2	6/29/92	Mosq	0.1235	0.1724	0.3307
51011	F-S-3	6/29/92	1 C var NBF	0.13025	0.1724	0.3307
51012	F-S-3	6/29/92	11 Mosq BF	0.1202	0.1724	0.3307
51013	F-C-3	6/29/92	3 C var BF	0.1417	0.1724	0.3307
51014	F-C-3	6/29/92	14 C var NBF	0.1825	0.1632	0.9652
51015	F-C-3	6/29/92	1 C other	0.319	0.393	1.335
51015B	F-C-3	6/29/92	1 C crep	0.367	0.409	1.489
51016	F-C-3	6/29/92	50 Mosq	0.1507	0.1632	0.9652
51017	F-S-4	6/29/92	Mosq	0.164	0.1632	0.9652
51018	F-C-4	6/29/92	6 C var BF	0.1607	0.1632	0.9652
51019	F-C-4	6/29/92	12 C var NBF	0.1455	0.1632	0.9652
51020	F-C-4	6/29/92	5 C other	0.121	0.1632	0.9652
51021	F-C-4	6/29/92	2 Mosq BF	0.179	0.1632	0.9652
51022	F-C-4	6/29/92	1 Mosq BF	0.187	0.1632	0.9652
51023	F-S-5	6/29/92	1 C other	0.177	0.1632	0.9652
51023B	F-S-5	6/29/92	2 C crep	0.305	0.409	1.489
51023E	F-S-5	6/29/92	1 C cock	0.289	0.409	1.489
51024	F-S-5	6/29/92	5 Mosq BF	0.1067	0.1632	0.9652
51025	F-C-5	6/29/92	5 C var BF	0.1215	0.1632	0.9652

51026	F-C-5	6/29/92	11 C var NBF	0.1105	0.1632	0.9652
51027	F-C-5	6/29/92	4 C other	ND	ND	ND
51027B	F-C-5	6/29/92	4 C crep	0.356	0.409	1.489
51027C	F-C-5	6/29/92	1 C var	0.308	0.409	1.489
51027D	F-C-5	6/29/92	1 C haem	0.327	0.409	1.489
51028	F-C-5	6/29/92	8 Mosq BF	0.1217	0.1632	0.9652
51029	F-C-4	7/1/92	1 C crep	0.1032	0.1632	0.9652
51030	F-C-3	7/1/92	1 C var BF	0.409	0.409	1.489
51031	F-C-3	7/1/92	1 C other	0.357	0.409	1.489
51031B	F-C-3	7/1/92	1 C crep	0.434	0.489	2.096
51032	F-S-5	7/1/92	1 C var NBF	0.09525	0.1632	0.9652
51033	F-S-3	7/1/92	13 Mosq	0.0952	0.1632	0.9652
51034	F-S-5	7/1/92	7 Mosq	0.1502	0.1632	0.9652
51035	F-C-2	7/1/92	16 Mosq	0.1505	0.1632	0.9652
51036	F-C-4	7/1/92	5 C var NBF	0.1465	0.1632	0.9652
51037	F-C-3	7/1/92	3 C var NBF	0.313	0.393	1.335
51038B	F-S-3	7/1/92	1 C crep	0.499	0.489	2.096
51039	F-C-4	7/1/92	1 C var BF	0.319	0.393	1.335
51040	F-S-2	7/1/92	1 C other	0.461	0.489	2.096
51041	F-C-2	7/1/92	2 C other	0.301	0.393	1.335
51041B	F-C-2	7/1/92	1 C crep	0.361	0.409	1.489
51042	F-C-1	7/1/92	6 C var NBF	0.422	0.393	1.335
51043	F-S-2	7/1/92	4 Mosq	0.474	0.489	2.096
51044B	F-C-1	7/1/92	1 C crep	0.369	0.409	1.489
51045	F-C-2	7/1/92	5 C var NBF	0.42	0.489	2.096
51046	F-S-1	7/1/92	5 C other	0.314	0.409	1.489
51047	F-C-4	7/1/92	20 Mosq	0.534	0.489	2.096
51048	F-C-3	7/1/92	37 Mosq	0.504	0.489	2.096
51049	F-C-3	7/1/92	33 Mosq	0.391	0.409	1.489
51050	F-C-1	7/6/92	2 C var NBF	0.343	0.471	1.521
51051	F-C-2	7/6/92	3 C crep NBF	0.179	0.2288	1.234
51051F	F-C-2	7/6/92	2 2-spot NBF	0.248	0.342	1.4345
51051G	F-C-2	7/6/92	2 2-spot BF	0.2323	0.342	1.4345
51051Z	F-C-2	7/6/92	2 Mangled NBF	0.263	0.2103	1.237
51052	F-C-2	7/6/92	5 Mosq BF	0.389	0.458	1.713
51052B	F-C-2	7/6/92	14 Mosq NBF	0.43	0.458	1.713
51053	F-C-2	7/6/92	14 C var NBF	0.381	0.471	1.521
51054	F-C-2	7/6/92	4 C var BF	0.399	0.471	1.521
51055A	F-C-3	7/6/92	10 Mosq BF	0.443	0.458	1.713
51055B	F-C-3	7/6/92	10 Mosq NBF	0.413	0.471	1.521
51056	F-C-3	7/6/92	4 C var BF	0.365	0.471	1.521
51057	F-C-3	7/6/92	1 C crep NBF	0.068	0.093	0.35
51058	F-C-4	7/6/92	11 Mosq BF	0.1438	0.2288	1.234
51058B	F-C-4	7/6/92	19 Mosq NBF	0.434	0.523	1.831
51059	F-C-4	7/6/92	10 C var NBF	0.367	0.471	1.521
51060	F-C-4	7/6/92	2 C var BF	0.361	0.471	1.521
51061	F-C-4	7/6/92	16 Mosq BF	0.376	0.523	1.831
51061B	F-C-4	7/6/92	17 Mosq NBF	0.429	0.523	1.831
51062	F-C-4	7/6/92	2 C mangled BF	0.1593	0.2242	1.225
51062B	F-C-4	7/6/92	2 C crep NBF	0.1403	0.2288	1.234

51094	A-S-4	7/20/92	2 C var NBF	0.2308	0.2579	1.387
51095	A-C-2	7/13/92	5 C var NBF	0.238	0.277	1.223
51096	A-C-5	7/13/92	1 C var NBF	0.274	0.277	1.223
51097	A-C-3	7/20/92	3 C var NBF	0.28	0.277	1.223
51098	A-S-5	7/15/92	2 C var NBF	0.277	0.277	1.223
51099	A-C-5	7/6/92	17 C var BF	0.278	0.277	1.223
51100	A-C-1	7/13/92	2 C var NBF	0.1655	0.2242	1.225
51101	A-C-2	7/13/92	1 C var BF	0.246	0.277	1.223
51102	A-C-1	7/20/92	1 C var BF	0.282	0.277	1.223
51103	A-S-3	7/20/92	1 C var NBF	0.286	0.277	1.223
51104	A-S-4	7/13/92	2 C var NBF	0.304	0.277	1.223
51105	A-C-4	7/13/92	1 C var NBF	0.326	0.277	1.223
51106	A-C-3	7/13/92	3 Mosq BF	0.141	0.093	0.35
51106B	A-C-3	7/13/92	13 Mosq NBF	0.083	0.085	0.231
51107	A-C-4	7/13/92	6 Mosq BF	0.125	0.093	0.35
51107B	A-C-4	7/13/92	12 Mosq NBF	0.318	0.342	1.4345
51108	A-C-3	7/20/92	10 Mosq BF	0.1635	0.2288	1.234
51108B	A-C-3	7/20/92	22 Mosq NBF	0.2915	0.342	1.4345
51109	A-C-3	7/20/92	1 C haem NBF	0.2205	0.342	1.4345
51110	A-S-1	7/20/92	1 C crep NBF	ND	ND	ND
51111	A-C-1	7/20/92	1 Mosq BF	0.263	0.342	1.4345
51112	A-S-3	7/6/92	2 Mosq BF	0.1883	0.2242	1.225
51113	A-C-5	7/13/92	1 C crep BF	0.394	0.421	1.06
51114	A-S-2	7/6/92	5 C var NBF	0.1225	0.11	0.384
51115	A-C-3	7/13/92	1 C crep NBF	0.082	0.093	0.35
51116	A-C-4	7/15/92	2 Mosq NBF	0.2455	0.342	1.4345
51117	A-C-5	7/15/92	1 C crep NBF	0.1755	0.2242	1.225
51118	A-C-5	7/6/92	15 Mosq BF	0.1675	0.2288	1.234
51118B	A-C-5	7/6/92	4 Mosq NBF	0.22025	0.2579	1.387
51119	A-C-3	7/15/92	1 Mosq NBF	0.2545	0.342	1.4345
51120	A-S-5	7/20/92	2 Mosq NBF	0.265	0.342	1.4345
51121	A-C-4	7/20/92	1 Mosq BF	0.3465	0.342	1.4345
51121B	A-C-4	7/20/92	8 Mosq NBF	ND	ND	ND
51122	A-S-4	7/13/92	8 Mosq NBF	0.087	0.099	0.371
51123	A-C-5	7/13/92	1 Mosq BF	0.092	0.093	0.35
51123B	A-C-5	7/13/92	3 Mosq NBF	0.101	0.135	0.302
51124	A-S-3	7/20/92	1 Mosq BF	ND	ND	ND
51124B	A-S-3	7/20/92	1 Mosq NBF	0.2332	0.268	1.523
51125	A-S-4	7/15/92	2 Mosq NBF	0.208	0.268	1.523
51126	A-S-4	7/6/92	2 Mosq NBF	0.117	0.099	0.371
51127	A-S-1	7/6/92	1 C var NBF	0.116	0.099	0.371
51128	A-S-3	7/6/92	2 C var NBF	0.078	0.099	0.371
51129	A-S-3	7/6/92	2 C var BF	0.506	0.489	2.096
51130	A-S-5	7/15/92	4 Mosq NBF	0.221	0.2579	1.387
51131	A-C-5	7/15/92	1 Mosq BF	ND	ND	ND
51131B	A-C-5	7/15/92	12 Mosq NBF	0.2475	0.268	1.523
51132	A-S-4	7/20/92	2 Mosq BF	0.242	0.2242	1.225
51132B	A-S-4	7/20/92	5 Mosq NBF	0.201	0.2242	1.225
51133	A-C-2	7/13/92	1 Mosq BF	0.252	0.2579	1.387
51133B	A-C-2	7/15/92	6 Mosq NBF	0.294	0.342	1.4345

51134	A-S-2	7/15/92	1 C crep NBF	0.1348	0.2242	1.225
51135	A-S-1	7/6/92	1 2-spot NBF	0.16	0.2242	1.225
51136	A-S-3	7/6/92	1 C crep NBF	0.228	0.342	1.4345
51136F	A-S-3	7/6/92	3 2-spot BF	0.2007	0.2288	1.234
51137	A-C-2	7/13/92	1 C crep NBF	0.1503	0.2288	1.234
51137F	A-C-2	7/13/92	1 2-spot NBF	0.305	0.342	1.345
51138	A-C-4	7/13/92	1 mangled NBF	0.132	0.2288	1.234
51139	A-S-4	7/13/92	1 C crep BF	0.214	0.342	1.4345
51139B	A-S-4	7/13/92	1 C crep NBF	0.208	0.268	1.523
51140	A-S-5	7/20/92	2 C crep BF	0.2595	0.342	1.4345
51141	A-S-4	7/6/92	1 C crep NBF	0.1955	0.268	1.523
51141F	A-S-4	7/6/92	1 2-spot NBF	0.1885	0.2103	1.237
51142	A-S-4	7/6/92	3 C var NBF	0.153	0.1555	0.965
51143	A-S-2	7/13/92	1 Mosq BF	0.1873	0.1555	0.965
51144	A-S-5	7/6/92	2 C var NBF	0.299	0.268	1.523
51445	A-S-1	7/13/92	1 C var NBF	0.238	0.268	1.523
51446	A-S-1	7/8/92	2 C crep NBF	0.107	0.11	0.384
51447	A-S-1	7/13/92	1 C crep NBF	ND	ND	ND
51148	A-S-3	7/13/92	1 Mosq BF	0.092	0.135	0.302
51148B	A-S-3	7/13/92	4 Mosq NBF	0.1	0.99	0.371
51149	A-S-2	7/6/92	2 C crep NBF	0.229	0.2242	1.225
51149Z	A-S-2	7/6/92	2 Mangled NBF	0.1955	0.2288	1.234
51149D	A-S-2	7/6/92	2 C haem NBF	0.2858	0.2579	1.387
51149F	A-S-2	7/6/92	5 2-spot NBF	0.2667	0.268	1.523
51149G	A-S-2	7/6/92	4 2-spot BF	0.2178	0.342	1.4345
51150	A-S-3	7/1/92	7 Mosq BF	0.2177	0.268	1.523
51150B	A-S-3	7/1/92	7 Mosq NBF	0.225	0.268	1.523
51151	A-C-3	7/6/92	14 C var BF	0.2075	0.2103	1.237
51152	A-C-2	7/6/92	2 C var BF	0.1853	0.1555	0.965
51153	A-C-1	7/6/92	2 C var BF	0.245	0.2103	1.237
51154	A-C-1	7/6/92	4 C var NBF	0.2507	0.2103	1.237
51155	A-C-4	7/6/92	15 C var NBF	0.215	0.2103	1.237
51156	A-C-2	7/6/92	8 C var NBF	0.2075	0.1555	0.965
51157	A-C-3	7/6/92	4 C var NBF	0.2205	0.268	1.523
51158	A-C-2	7/6/92	3 Mosq BF	0.1905	0.2103	1.237
51158B	A-C-2	7/6/92	1 Mosq NBF	0.1943	0.2103	1.237
51159	A-S-2	7/1/92	6 Mosq NBF	0.202	0.268	1.523
51160	A-C-5	7/6/92	1 C crep NBF	0.1797	0.268	1.523
51161	A-C-2	7/6/92	1 C crep NBF	0.2247	0.268	1.523
51162	A-S-4	7/1/92	1 2-spot BF	0.2747	0.268	1.523
51163	A-C-1	7/6/92	1 C haem NBF	0.1402	0.1555	0.965
51163Z	A-C-1	7/6/92	1 Mangled NBF	0.1787	0.268	1.523
51164	A-C-3	7/6/92	2 C crep NBF	0.185	0.2103	1.237
51164B	A-C-3	7/6/92	1 C crep BF	0.1897	0.2103	1.237
51165	A-C-1	7/6/92	3 Mosq BF	0.1605	0.1555	0.965
51166	A-C-4	7/6/92	15 Mosq BF	0.092	0.093	0.35
5166B	A-C-4	7/6/92	12 Mosq NBF	0.206	0.2103	1.237
51167	A-C-3	7/6/92	17 Mosq BF	0.2307	0.2242	1.225
51167B	A-C-3	7/6/92	3 Mosq NBF	0.2205	0.2579	1.387
51168	A-S-3	6/29/92	14 Mosq BF	0.261	0.144	0.423

51168B	A-S-3	6/29/92	8 Mosq NBF	0.2195	0.2579	1.387
51169	A-C-1	7/1/92	3 C var NBF	0.1653	0.2288	1.234
51170	A-C-1	7/1/92	6 C var NBF	0.2685	0.2288	1.234
51171	A-C-3	6/29/92	2 C var NBF	0.1653	0.2242	1.225
51172	A-C-4	7/6/92	23 C var BF	0.1525	0.2288	1.234
51173	A-S-1	6/29/92	1 Mosq BF	0.3462	0.342	1.345
51174	A-S-1	7/1/92	1 C var NBF	0.2458	0.2579	1.387
51175	A-S-3	6/29/92	1 C var NBF	0.1703	0.2242	1.225
51176	A-C-3	7/1/92	1 C var NBF	0.193	0.2579	1.387
51177	A-C-3	6/29/92	1 C crep NBF	0.2365	0.2242	1.225
51178	A-S-1	7/1/92	2 C crep NBF	0.1755	0.2288	1.234
51179	A-C-3	6/29/92	7 Mosq BF	0.2045	0.2288	1.234
51179B	A-C-3	6/29/92	6 Mosq NBF	0.262	0.342	1.4345
51180	A-C-4	6/29/92	2 Mosq NBF	0.074	0.18	0.367
51180B	A-C-4	6/29/92	1 Mosq BF	0.2463	0.342	1.4345
51181	A-C-3	7/1/92	15 Mosq BF	0.237	0.2242	1.225
51181B	A-C-3	7/1/92	12 Mosq NBF	0.21325	0.2242	1.225
51182	A-C-4	7/1/92	2 Mosq BF	0.251	0.268	1.523
51182B	A-C-4	7/1/92	5 Mosq NBF	0.23	0.2579	1.387
51183	A-S-4	7/1/92	7 Mosq NBF	0.191	0.2288	1.234
51184	A-C-5	7/1/92	2 Mosq BF	0.2205	0.2242	1.225
51184B	A-C-5	7/1/92	2 Mosq NBF	0.1717	0.2288	1.234
51185	A-C-2	7/1/92	2 Mosq BF	0.2493	0.2579	1.387
51185B	A-C-2	7/1/92	3 Mosq NBF	0.184	0.2103	1.237
51186	A-C-5	7/1/92	2 2-spot NBF	0.192	0.2103	1.237
51186G	A-C-5	7/1/92	1 2-spot BF	0.108	0.135	0.302
51186Z	A-C-5	7/1/92	1 Mangled BF	ND	ND	ND
51187	A-C-1	7/1/92	3 C crep NBF	0.148	0.2288	1.234
51187G	A-C-1	7/1/92	1 2-spot NBF	0.292	0.342	1.4345
51188	A-C-4	7/6/92	3 2-spot NBF	0.061	0.085	0.231
51188B	A-C-4	7/6/92	2 C crep NBF	0.1762	0.268	1.523
51188Y	A-C-4	7/6/92	1 Clear Wing BF	0.099	0.135	0.302
51188G	A-C-4	7/6/92	2 2-spot BF	0.1755	0.1555	0.965
51189	F-C-3	7/8/92	9 Mosq BF	0.162	0.2242	1.225
51189B	F-C-3	7/8/92	10 Mosq NBF	0.2132	0.268	1.523
51190	F-C-3	7/18/92	1 C var NBF	0.1665	0.1555	0.965
51191	F-C-2	7/13/92	1 C var NBF	0.2127	0.2242	1.225
51192	F-S-5	7/13/92	2 Mosq NBF	0.2173	0.2579	1.387
51193	F-C-2	7/8/92	2 C var BF	0.161	0.2103	1.237
51194	F-C-4	7/8/92	1 C var BF	0.21	0.18	0.367
51195	F-C-1	7/13/92	2 C var NBF	0.157	0.2103	1.237
51196	F-C-1	7/13/92	2 C var BF	0.268	0.342	1.4345
51197	F-C-2	7/8/92	4 C var NBF	0.228	0.2288	1.234
51198	F-C-3	7/8/92	1 C var NBF	0.1857	0.268	1.523
51199	F-C-4	7/8/92	4 C var NBF	0.2718	0.2579	1.387
51200	F-C-3	7/13/92	2 C var NBF	0.1695	0.1555	0.965
51201	F-C-1	7/13/92	1 Mosq BF	0.1795	0.1555	0.965
51202	F-C-4	7/8/92	5 Mosq BF	0.091	0.093	0.35
51202B	F-C-4	7/8/92	3 Mosq NBF	0.1	0.099	0.371
51203	F-C-2	7/8/92	14 Mosq BF	0.107	0.11	0.384

51203B	F-C-2	7/8/92	10 Mosq NBF	0.086	0.093	0.35
51204	F-C-3	7/13/92	29 Mosq BF	0.1225	0.093	0.35
51204B	F-C-3	7/13/92	21 Mosq NBF	0.093	0.093	0.35
51205	F-C-5	7/8/92	1 C crep BF	0.1775	0.1555	0.965
51205D	F-C-5	7/8/92	1 C haem BF	0.246	0.268	1.523
51206	F-C-4	7/8/92	1 C crep BF	0.2105	0.18	0.367
51207	F-S-1	7/13/92	2 C crep BF	0.1822	0.1555	0.965
51208	F-S-2	7/13/92	11 Mosq NBF	0.1255	0.135	0.302
51209	F-S-3	7/13/92	2 Mosq BF	0.105	0.135	0.302
51209B	F-S-3	7/13/92	22 Mosq NBF	0.188	0.2103	1.237
51210	F-S-4	7/13/92	2 Mosq BF	0.1085	0.11	0.384
51210B	F-S-4	7/13/92	5 Mosq NBF	ND	ND	ND
51211	F-C-5	7/8/92	5 Mosq BF	0.1972	0.18	0.367
51211B	F-C-5	7/8/92	3 Mosq NBF	0.08	0.11	0.384
51212	F-C-2	7/13/92	43 Mosq BF	ND	ND	ND
51212B	F-C-2	7/13/92	17 Mosq NBF	ND	ND	ND
51213	F-C-3	7/13/92	37 Mosq BF	0.202	0.18	0.367
51213B	F-C-3	7/13/92	26 Mosq NBF	ND	ND	ND
51214	F-C-2	7/8/92	15 Mosq BF	0.11	0.135	0.302
51214B	F-C-2	7/8/92	15 Mosq NBF	ND	ND	ND
51215	F-S-3	6/29/92	8 2-spot BF	0.097	0.11	0.384
51216	F-C-5	7/13/92	7 Mosq BF	0.2	0.18	0.367
51216B	F-C-5	7/13/92	9 Mosq NBF	0.1695	0.2103	1.237
51217	F-C-1	7/15/92	2 Mosq NBF	0.1665	0.2103	1.237
51218	F-C-1	7/15/92	2 C var NBF	0.1407	0.1555	0.965
51219	F-C-2	7/15/92	7 C var NBF	0.1932	0.2103	1.237
51220	F-C-2	7/15/92	3 C var BF	0.093	0.093	0.35
51221	F-C-1	7/15/92	2 C var BF	ND	ND	ND
51222	F-S-2	7/15/92	7 C var NBF	0.083	0.099	0.371
51223	F-C-3	7/13/92	2 C var BF	0.094	0.135	0.302
51224	F-S-3	7/15/92	2 Mosq BF	0.19	0.2103	1.237
51224B	F-S-3	7/15/92	23 Mosq NBF	0.1683	0.2103	1.237
51225	F-C-2	7/15/92	4 Mosq BF	0.109	0.093	0.35
51225B	F-C-2	7/15/92	2 Mosq NBF	0.105	0.135	0.302
51226	F-S-5	7/15/92	6 Mosq NBF	0.113	0.11	0.384
51227	F-S-4	7/15/92	4 Mosq NBF	0.1752	0.18	0.367
51228	F-S-2	7/15/92	2 Mosq BF	0.103	0.093	0.35
51228B	F-S-2	7/15/92	2 Mosq NBF	0.101	0.135	0.302
51229	F-C-4	7/13/92	29 Mosq BF	0.117	0.107	0.383
51229B	F-C-4	7/13/92	16 Mosq NBF	0.094	0.099	0.371
51230	F-S-2	7/15/92	1 C crep BF	0.099	0.099	0.371
51231	F-C-5	7/13/92	1 C crep NBF	0.1735	0.18	0.367
51231C	F-C-5	7/13/92	1 C var NBF	0.057	0.085	0.231
51232	F-S-1	7/15/92	2 C crep NBF	0.112	0.11	0.384
51233	F-C-2	7/15/92	1 C crep NBF	0.26	0.361	1.19
51234	F-C-4	7/20/92	1 C var BF	0.108	0.099	0.371
51235	F-C-3	7/20/92	1 C var NBF	0.099	0.135	0.302
51236	F-C-4	7/15/92	3 C var NBF	0.097	0.135	0.302
51237	F-C-1	7/20/92	5 C var NBF	ND	ND	ND
51238	F-C-2	7/20/92	2 C var NBF	ND	ND	ND

51239	F-S-2	7/20/92	1 C var NBF	0.093	0.093	0.35
51240	F-S-1	7/20/92	1 C var NBF	ND	ND	ND
51241	F-S-3	7/20/92	2 C var NBF	0.303	0.322	1.098
51242	F-C-3	7/15/92	11 C var NBF	0.1027	0.093	0.35
51243	F-C-1	7/20/92	4 C var BF	0.107	0.18	0.367
51244	F-C-5	7/20/92	1 C var NBF	0.082	0.099	0.371
51245	F-S-2	7/20/92	1 Mosq NBF	0.072	0.093	0.35
51246	F-C-5	7/15/92	1 C crep NBF	0.303	0.421	1.06
51246D	F-C-5	7/15/92	1 C haem BF	0.336	0.421	1.06
51247	F-S-3	7/15/92	1 C crep NBF	0.378	0.458	1.305
51247B	F-S-3	7/15/92	1 C crep BF	0.355	0.421	1.06
51248	F-S-2	7/20/92	1 C crep NBF	0.406	0.458	1.305
51249	F-S-1	7/20/92	1 C crep NBF	0.309	0.421	1.06
51249B	F-S-1	7/20/92	1 C crep BF	0.114	0.099	0.371
51250	F-S-4	7/13/92	1 C crep NBF	0.244	0.352	0.728
51250B	F-S-4	7/13/92	1 C crep BF	0.099	0.099	0.371
51251	F-S-2	7/13/92	2 C crep BF	0.094	0.135	0.302
51251B	F-S-2	7/13/92	1 C crep NBF	0.087	0.099	0.371
51252	F-S-4	7/15/92	2 C crep BF	0.2791	0.352	0.728
51252B	F-S-4	7/15/92	1 C crep NBF	0.106	0.093	0.35
51253	F-C-3	7/13/92	1 C crep NBF	0.268	0.322	1.098
51253Z	F-C-3	7/13/92	1 Mangled BF	0.346	0.361	1.19
51254	F-S-5	7/13/92	1 C crep BF	0.318	0.322	1.098
51254B	F-S-5	7/13/92	2 C crep NBF	0.34	0.421	1.06
51254D	F-S-5	7/13/92	1 C haem NBF	0.269	0.322	1.098
51254Z	F-S-5	7/13/92	1 Mangled BF	0.265	0.352	0.728
51255	F-C-1	7/13/92	2 C crep BF	0.345	0.421	1.06
51255B	F-C-1	7/13/92	2 C crep NBF	0.24	0.18	0.367
51255Z	F-C-1	7/13/92	1 Mangled BF	ND	ND	ND
51256	F-C-3	7/15/92	16 Mosq BF	0.245	0.144	0.423
51256B	F-C-3	7/15/92	22 Mosq NBF	0.359	0.352	0.728
51257	F-C-4	7/15/92	13 Mosq BF	0.206	0.144	0.423
51257B	F-C-4	7/15/92	16 Mosq NBF	ND	ND	ND
51258	F-C-5	7/15/92	4 Mosq BF	0.129	0.144	0.423
51258B	F-C-5	7/15/92	9 Mosq NBF	0.341	0.445	0.852
51259	F-C-3	7/15/92	20 Mosq BF	0.178	0.144	0.423
51259B	F-C-3	7/15/92	10 Mosq NBF	ND	ND	ND
51260	F-S-1	7/22/92	2 C var NBF	0.094	0.099	0.371
51261	F-C-2	7/22/92	2 C var BF	ND	ND	ND
51262	F-C-3	7/22/92	1 C var NBF	0.06	0.085	0.231
51263	F-C-1	7/22/92	14 C var BF	ND	ND	ND
51264	F-C-2	7/22/92	3 C var NBF	0.308	0.361	1.19
51265	F-C-3	7/22/92	2 C var BF	0.116	0.099	0.371
51266	F-C-1	7/22/92	7 C var NBF	0.104	0.099	0.371
51267	F-C-2	7/20/92	5 C var BF	0.212	0.18	0.367
51268	F-C-1	7/20/92	1 Mosq BF	0.097	0.135	0.302
51269	F-S-1	7/22/92	1 Mosq NBF	0.105	0.135	0.302
51270	F-C-1	7/22/92	1 Mosq BF	0.157	0.18	0.367
51270B	F-C-1	7/22/92	1 Mosq NBF	0.083	0.099	0.371
51271	F-S-2	7/27/92	5 Mosq NBF	0.2285	0.18	0.367

51272	F-S-4	7/20/92	1 Mosq BF	0.071	0.085	0.231
51272B	F-S-4	7/20/92	2 Mosq NBF	0.084	0.093	0.35
51273	F-C-5	7/20/92	3 Mosq BF	0.096	0.11	0.384
51273B	F-C-5	7/20/92	11 Mosq NBF	0.092	0.18	0.367
51274	F-S-3	7/20/92	1 Mosq BF	ND	ND	ND
51274B	F-S-3	7/20/92	15 Mosq NBF	0.085	0.093	0.35
51275	F-C-3	7/22/92	1 C crep NBF	0.093	0.093	0.35
51276	F-S-4	7/20/92	1 C crep NBF	0.216	0.18	0.367
51277	F-S-2	7/22/92	2 C crep NBF	0.081	0.11	0.384
51278	F-S-1	7/22/92	2 C crep NBF	0.108	0.135	0.302
51278B	F-S-1	7/22/92	1 C crep BF	0.078	0.085	0.231
51279	F-S-1	7/27/92	2 C crep NBF	ND	ND	ND
51279B	F-S-1	7/27/92	1 C crep BF	0.355	0.352	0.728
51280	F-S-3	7/27/92	1 C crep NBF	0.086	0.11	0.384
51280B	F-S-3	7/27/92	1 C haem NBF	0.102	0.11	0.384
51281	F-C-1	7/22/92	2 crep BF	0.111	0.18	0.367
51281B	F-C-1	7/22/92	1 C crep NBF	0.163	0.18	0.367
51281Z	F-C-1	7/22/92	1 Mangled NBF	0.318	0.361	1.19
51282	F-C-2	8/5/92	5 C var NBF	0.374	0.421	1.06
51283	F-S-5	8/5/92	1 C var NBF	0.32	0.361	1.19
51284	F-C-1	8/5/92	2 C var BF	0.328	0.361	1.19
51285	F-S-1	8/5/92	1 C var NBF	0.274	0.352	0.728
51286	F-C-2	8/5/92	2 C var BF	0.259	0.421	1.06
51287	F-C-3	8/5/92	1 C var NBF	0.265	0.361	1.19
51288	F-S-2	8/5/92	1 Mosq BF	0.483	0.352	0.728
51288B	F-S-2	8/5/92	2 Mosq NBF	0.318	0.361	1.19
51289	F-S-4	7/27/92	2 Mosq BF	0.357	0.352	0.728
51289B	F-S-4	7/27/92	15 Mosq NBF	0.322	0.361	1.19
51290	F-C-3	7/20/92	17 Mosq BF	0.213	0.144	0.423
51290B	F-C-3	7/20/92	35 Mosq NBF	0.309	0.361	1.19
51291	F-C-4	7/20/92	3 Mosq BF	0.113	0.109	0.271
51291B	F-C-4	7/20/92	16 Mosq NBF	0.33	0.322	1.098
51292	F-C-2	7/20/92	19 Mosq BF	0.426	0.458	1.305
51292B	F-C-2	7/20/92	24 Mosq NBF	0.353	0.379	1.224
51293	F-C-2	7/22/92	14 Mosq BF	0.213	0.144	0.423
51293B	F-C-2	7/22/92	6 Mosq NBF	0.361	0.361	1.19
51293C	F-C-2	7/22/92	1 C var NBF	0.258	0.361	1.19
51294	F-C-2	8/5/92	7 Mosq BF	0.3625	0.352	0.728
51294B	F-C-2	8/5/92	1 Mosq NBF	0.282	0.361	1.19
51295	F-S-2	7/22/92	11 Mosq NBF	0.339	0.458	1.305
51295B	F-S-2	7/22/92	2 Mosq BF	0.301	0.352	0.728
51296	F-C-4	8/5/92	45 Mosq BF	0.297	0.361	1.19
51296B	F-C-4	8/5/92	4 Mosq NBF	0.3188	0.352	0.728
51297	F-C-3	7/20/92	21 Mosq BF	0.161	0.109	0.271
51297B	F-C-3	7/20/92	51 Mosq NBF	0.308	0.379	1.224
51298	F-C-3	8/5/92	52 Mosq BF	0.459	0.445	1.136
51298B	F-C-3	8/5/92	10 Mosq NBF	0.289	0.352	0.728
51299	F-C-2	8/5/92	1 C crep NBF	0.349	0.379	1.224
51300	F-S-2	8/5/92	1 C crep NBF	0.311	0.361	1.19
51301	F-C-5	7/20/92	2 C crep BF	0.366	0.458	1.305

51302	F-S-5	8/5/92	2 C crep NBF	0.299	0.361	1.19
51303	F-S-4	8/5/92	1 C crep BF	0.336	0.421	1.06
51303B	F-S-4	8/5/92	2 C crep NBF	0.332	0.421	1.06
51304	F-S-2	7/27/92	2 C crep BF	0.33	0.361	1.19
51304B	F-S-2	7/27/92	5 C crep NBF	0.341	0.421	1.06
51305	F-S-3	7/22/92	3 C crep BF	0.337	0.421	1.06
51305B	F-S-3	7/22/92	3 C crep NBF	0.289	0.352	0.728
51306	F-S-3	8/5/92	10 Mosq BF	0.475	0.352	0.728
51306B	F-S-3	8/5/92	42 Mosq NBF	0.299	0.322	1.098
51307	F-S-3	7/27/92	1 Mosq BF	0.272	0.361	1.19
51307B	F-S-3	7/27/92	31 Mosq NBF	0.344	0.379	1.224
51308	F-S-3	7/27/92	2 Mosq BF	0.3925	0.458	1.305
51308B	F-S-3	7/27/92	27 Mosq NBF	0.343	0.379	1.224
51309	F-S-3	7/22/92	1 Mosq BF	0.328	0.458	1.305
51309B	F-S-3	7/22/92	41 Mosq NBF	0.278	0.322	1.098
51310	F-C-3	7/22/92	23 Mosq BF	0.5615	0.458	1.305
51310B	F-C-3	7/22/92	25 Mosq NBF	0.353	0.379	1.224
51311	F-C-3	7/22/92	20 Mosq BF	0.324	0.322	1.098
51311B	F-C-3	7/22/92	28 Mosq NBF	0.355	0.379	1.224
51312	F-S-5	8/5/92	13 Mosq BF	0.7065	0.379	1.224
51312B	F-S-5	8/5/92	35 Mosq NBF	0.245	0.361	1.19
51313	F-S-4	8/5/92	6 Mosq BF	0.258	0.458	1.305
51313B	F-S-4	8/5/92	54 Mosq NBF	0.409	0.458	1.305
51314	F-S-3	7/22/92	6 Mosq BF	0.318	0.352	0.728
51314B	F-S-3	7/22/92	44 Mosq NBF	0.249	0.352	0.728
51315	F-C-4	8/5/92	42 Mosq BF	0.353	0.458	1.305
51315B	F-C-4	8/5/92	9 Mosq NBF	0.094	0.144	0.423
51316	F-S-4	8/5/92	10 Mosq BF	0.44	0.458	1.305
51316B	F-S-4	8/5/92	61 Mosq NBF	0.2521	0.3791	1.224
51317	F-C-3	8/5/92	65 Mosq BF	0.76175	0.458	1.305
51317B	F-C-3	8/5/92	7 Mosq NBF	0.335	0.361	1.19
51318	A-C-3	8/5/92	6 Mosq BF	0.389	0.463	1.117
51318B	A-C-3	8/5/92	2 Mosq NBF	0.3105	0.379	1.224
51319	A-C-2	8/5/92	1 C var NBF	0.261	0.421	1.06
51320	A-C-2	8/12/92	1 C var NBF	0.284	0.421	1.06
51321	A-S-5	8/10/92	1 C var NBF	0.309	0.458	1.305
51322	A-C-5	8/10/92	5 C var BF	0.374	0.421	1.06
51323	A-C-4	8/10/92	9 C var NBF	0.086	0.08	0.285
51324	A-S-1	8/10/92	6 Mosq BF	0.22	0.2	0.855
51324B	A-S-1	8/10/92	2 Mosq NBF	0.236	0.209	0.855
51325	A-C-3	8/10/92	2 C var NBF	0.354	0.322	1.098
51326	A-C-5	8/10/92	4 C var NBF	0.316	0.352	0.728
51327	A-C-5	8/5/92	1 C var NBF	0.2182	0.209	0.855
51328	A-S-4	8/10/92	2 C var NBF	0.329	0.322	1.098
51329	A-C-2	8/10/92	2 C var BF	0.286	0.322	1.098
51330	A-C-3	8/10/92	3 C var BF	0.352	0.421	1.06
51331	A-C-2	8/10/92	7 C var NBF	0.354	0.458	1.305
51332	A-C-4	8/10/92	3 C var BF	0.259	0.322	1.098
51333	A-C-1	8/5/92	11 Mosq BF	0.2047	0.178	0.692
51333B	A-C-1	8/5/92	1 Mosq NBF	0.079	0.144	0.423

51334	A-S-2	8/5/92	9 Mosq BF	0.15	0.144	0.423
51334B	A-S-2	8/5/92	2 Mosq NBF	0.292	0.463	1.117
51335	A-S-3	8/5/92	11 Mosq BF	0.423	0.445	1.136
51335B	A-S-3	8/5/92	7 Mosq NBF	0.286	0.322	1.098
51336	A-C-2	8/5/92	9 Mosq BF	0.146	0.144	0.423
51336B	A-C-2	8/5/92	2 Mosq NBF	0.292	0.445	1.136
51337	A-S-2	8/10/92	2 Mosq BF	0.205	0.178	0.692
51338	A-C-1	8/10/92	6 Mosq BF	0.157	0.144	0.423
51339	A-C-2	8/12/92	2 Mosq BF	0.214	0.144	0.423
51339B	A-C-2	8/12/92	12 Mosq NBF	0.274	0.361	1.19
51340	A-S-3	8/10/92	9 Mosq BF	0.159	0.109	0.271
51340B	A-S-3	8/10/92	11 Mosq NBF	0.253	0.445	1.136
51341	A-S-5	8/10/92	6 Mosq BF	0.455	0.463	1.117
51341B	A-S-5	8/10/92	14 Mosq NBF	0.241	0.322	1.098
51342	A-C-2	8/10/92	11 Mosq BF	0.12	0.144	0.423
51342B	A-C-2	8/10/92	4 Mosq NBF	0.311	0.421	1.06
51343	A-S-4	8/5/92	4 Mosq BF	0.172	0.144	0.423
51343B	A-S-4	8/5/92	6 Mosq NBF	0.328	0.352	0.728
51344	A-S-5	8/5/92	4 Mosq BF	0.337	0.463	1.117
51344B	A-S-5	8/5/92	11 Mosq NBF	0.263	0.322	1.098
51345	A-S-1	8/5/92	5 Mosq BF	0.157	0.109	0.271
51345B	A-S-1	8/5/92	4 Mosq NBF	0.275	0.322	1.098
51346	A-C-5	8/5/92	12 Mosq BF	0.409	0.458	1.305
51346B	A-C-5	8/5/92	10 Mosq NBF	0.266	0.445	1.136
51347	A-S-4	8/10/92	12 Mosq BF	0.173	0.144	0.423
51347B	A-S-4	8/10/92	26 Mosq NBF	0.343	0.458	1.305
51348	A-C-5	8/10/92	23 Mosq BF	0.308	0.322	1.098
51349	A-S-5	8/10/92	3 C crep BF	0.339	0.352	0.728
51350	A-S-2	8/10/92	4 C crep NBF	0.363	0.458	1.305
51351	A-C-5	8/10/92	2 C crep BF	0.315	0.352	0.728
51351B	A-C-5	8/10/92	1 C crep NBF	0.301	0.463	1.117
51351Z	A-C-5	8/10/92	7 Mangled NBF	0.322	0.421	1.06
51351D	A-C-5	8/10/92	5 C haem BF	0.31	0.421	1.06
51351Y	A-C-5	8/10/92	6 Mangled BF	0.322	0.421	1.06
51351E	A-C-5	8/10/92	3 C haem NBF	0.363	0.379	1.224
51352	A-C-4	8/10/92	51 Mosq BF	0.2841	0.144	0.423
51352B	A-C-4	8/10/92	40 Mosq NBF	0.3285	0.379	1.224
51353	A-S-1	8/10/92	3 Mangled	0.34	0.458	1.305
51354	A-C-3	8/12/92	17 Mosq BF	0.5315	0.379	1.224
51354B	A-C-3	8/12/92	26 Mosq NBF	0.327	0.445	1.136
51355	A-C-4	8/10/92	1 C var BF	0.108	0.119	0.4357
51355B	A-C-4	8/10/92	4 Mangled	0.105	0.122	0.438
51356	A-S-4	8/10/92	2 C crep BF	0.1972	0.209	0.855
51356D	A-S-4	8/10/92	3 C haem NBF	0.1197	0.144	0.543
51356Z	A-S-4	8/10/92	2 Mangled	0.228	0.209	0.855
51357	A-C-3	8/10/92	61 Mosq BF	0.1445	0.144	0.543
51357B	A-C-3	8/10/92	23 Mosq NBF	0.124	0.122	0.438
51358	F-S-3	8/10/92	12 Mosq NBF	0.464	0.463	1.117
51359	A-C-1	8/17/92	2 Mosq BF	0.164	0.144	0.423
51360	A-S-1	8/17/92	1 Mosq BF	0.371	0.445	1.136

51361	A-S-5	8/17/92	1 Mosq BF	0.213	0.322	1.098
51362	A-S-2	8/17/92	2 Mosq NBF	0.25	0.445	1.136
51363	A-C-1	8/17/92	1 C var BF	0.291	0.463	1.117
51364	A-C-3	8/17/92	2 C var NBF	0.295	0.463	1.117
51365	F-S-4	8/10/92	1 C var NBF	0.369	0.463	1.117
51366	F-C-5	8/5/92	6 C var BF	0.285	0.445	1.136
51367	F-C-5	8/5/92	1 C var NBF	0.244	0.209	1.855
51368	A-C-5	8/12/92	4 C var NBF	0.339	0.463	1.117
51369	A-C-2	8/12/92	2 C var BF	0.366	0.463	1.117
51370	A-C-3	8/12/92	1 C var NBF	0.359	0.463	1.117
51371	A-C-5	8/12/92	1 C var BF	0.324	0.463	1.117
51372	A-C-4	8/12/92	1 C var NBF	0.286	0.445	1.136
51373	F-S-2	8/10/92	8 Mosq NBF	0.267	0.445	1.136
51374	A-S-3	8/17/92	2 Mosq BF	0.333	0.445	1.136
51374B	A-S-3	8/17/92	19 Mosq NBF	0.272	0.445	1.136
51375	F-C-5	8/5/92	29 Mosq BF	0.387	0.445	1.136
51375B	F-C-5	8/5/92	5 Mosq NBF	0.301	0.463	1.117
51376	F-C-2	8/10/92	1 Mosq BF	0.301	0.463	1.117
51376B	F-C-2	8/10/92	8 Mosq NBF	0.303	0.445	1.136
51377	F-S-4	8/10/92	1 Mosq BF	0.279	0.463	1.117
51377B	F-S-4	8/10/92	???	0.296	0.463	1.117
51378	A-C-3	8/17/92	14 Mosq BF	0.1787	0.178	0.692
51378B	A-C-3	8/17/92	44 Mosq NBF	0.208	0.178	0.692
51379	A-S-3	8/12/92	4 Mosq BF	0.113	0.122	0.438
51379B	A-S-3	8/12/92	20 Mosq NBF	0.234	0.178	0.692
51380	A-C-5	8/17/92	1 C var NBF	0.218	0.209	0.855
51381	A-C-2	8/17/92	3 Mosq BF	0.1282	0.144	0.543
51381B	A-C-2	8/17/92	1 Mosq NBF	0.121	0.122	0.438
51382	A-S-2	8/12/92	4 Mosq NBF	0.099	0.122	0.438
51383	A-S-4	8/12/92	2 Mosq BF	0.112	0.119	0.4357
51383B	A-S-4	8/12/92	16 Mosq NBF	0.1607	0.144	0.543
51384	A-C-5	8/17/92	2 Mosq BF	0.217	0.178	0.855
51384B	A-C-5	8/17/92	15 Mosq NBF	0.1427	0.144	0.543
51385	A-S-5	8/12/92	8 Mosq NBF	0.126	0.122	0.438
51386	F-C-4	8/10/92	9 Mosq BF	0.1972	0.178	0.692
51386B	F-C-4	8/10/92	11 Mosq NBF	0.111	0.122	0.438
51387	A-C-5	8/12/92	5 Mosq BF	0.115	0.117	0.4357
51387B	A-C-5	8/12/92	32 Mosq NBF	0.112	0.085	0.231
51388	A-S-4	8/17/92	1 Mosq BF	0.102	0.122	0.438
51388B	A-S-4	8/17/92	19 Mosq NBF	0.208	0.178	0.692
51389	F-C-3	8/10/92	31 Mosq BF	0.227	0.209	0.855
51389B	F-C-3	8/10/92	13 Mosq NBF	0.134	0.122	0.438
51390	F-C-4	8/5/92	45 Mosq BF	0.109	0.119	0.4357
51390B	F-C-4	8/5/92	4 Mosq NBF	0.113	0.122	0.438
51391	A-S-1	8/17/92	1 Mangled	0.1322	0.144	0.543
51392	A-C-2	8/17/92	1 C haem BF	0.188	0.209	0.855
51393	F-S-2	8/10/92	1 Mangled BF	0.18	0.178	0.692
51394	A-S-5	8/17/92	1 C crep BF	0.096	0.119	0.4357
51394B	A-S-5	8/17/92	2 C crep NBF	0.178	0.178	0.692
51395	A-S-3	8/12/92	1 C crep NBF	0.15	0.144	0.543

51396	A-S-2	8/12/92	1 C crep BF	0.207	0.209	1.855
51397	F-C-4	8/5/92	1 C cock BF	0.106	0.122	0.438
51398	A-C-5	8/17/92	3 C crep NBF	0.112	0.122	0.438
51399	F-C-5	8/5/92	1 C crep BF	0.237	0.209	0.855
51399B	F-C-5	8/5/92	2 C crep NBF	0.141	0.144	0.543
51399D	F-C-5	8/5/92	1 C haem BF	0.1	0.119	0.4357
51399Z	F-C-5	8/5/92	1 Mangled BF	0.1312	0.144	0.543
51400	A-C-4	8/17/92	7 Mosq BF	0.155	0.144	0.543
51400B	A-C-4	8/17/92	32 Mosq NBF	0.12	0.119	0.4357
51401	A-C-4	8/12/92	4 Mosq BF	0.097	0.122	0.438
51401B	A-C-4	8/12/92	20 Mosq NBF	0.157	0.144	0.543
51402	F-C-4	8/5/92	47 Mosq BF	0.206	0.178	0.692
51402B	F-C-4	8/5/92	3 Mosq NBF	0.1397	0.144	0.543
51403	F-C-4	8/5/92	47 Mosq BF	0.111	0.119	0.4357
51403B	F-C-4	8/5/92	3 Mosq NBF	0.098	0.122	0.438
51404	F-C-5	8/5/92	39 Mosq BF	0.137	0.144	0.543
51404B	F-C-5	8/5/92	11 Mosq NBF	0.12	0.122	0.438
51405	F-C-5	8/5/92	32 Mosq BF	0.131	0.122	0.438
51405B	F-C-5	8/5/92	17 Mosq NBF	0.119	0.119	0.4357
51406	F-C-4	8/5/92	58 Mosq BF	0.157	0.119	0.4357
51406B	F-C-4	8/5/92	6 Mosq NBF	0.118	0.119	0.4357
51407	F-C-4	8/5/92	38 Mosq BF	0.1467	0.144	0.543
51407B	F-C-4	8/5/92	12 Mosq NBF	0.1825	0.178	0.692
51408	A-C-3	7/29/92	6 C var NBF	0.1925	0.209	0.855
51409	A-C-1	8/3/92	1 C var BF	0.102	0.119	0.4357
51410	A-S-2	8/3/92	3 C var NBF	0.103	0.199	0.4357
51411	A-C-5	7/29/92	3 C var NBF	0.125	0.122	0.438
51412	A-S-5	7/29/92	1 C var NBF	0.1125	0.119	0.4357
51413	A-C-2	7/27/92	1 C var NBF	0.095	0.122	0.438
51414	A-C-4	7/20/92	2 C var BF	0.186	0.209	0.855
51415	A-C-4	8/3/92	6 C var NBF	0.2167	0.178	0.692
51416	A-C-2	8/3/92	1 C var NBF	0.101	0.199	0.4357
51417	A-C-3	7/29/92	2 C var NBF	0.1707	0.178	0.692
51418	A-C-4	7/22/92	4 C var BF	0.099	0.122	0.438
51419	A-S-1	7/29/92	2 C var NBF	0.1487	0.178	0.692
51420	A-C-2	7/29/92	2 C var NBF	0.1695	0.178	0.692
51421	A-C-3	8/3/92	5 C var NBF	0.1985	0.178	0.692
51422	A-C-1	7/22/92	2 C var NBF	0.211	0.178	0.692
51423	A-C-1	7/27/92	2 C var BF	0.108	0.119	0.4357
51424	A-C-3	8/3/92	2 C var BF	0.093	0.119	0.4357
51425	A-C-1	7/22/92	6 C var BF	0.2462	0.209	0.855
51426	A-C-5	7/27/92	1 C var BF	0.1592	0.144	0.543
51427	A-S-4	7/29/92	2 C var NBF	0.112	0.119	0.4357
51428	A-C-4	8/3/92	3 C var BF	0.23	0.209	0.855
51429	A-C-1	7/27/92	2 C var NBF	0.1005	0.122	0.438
51430	A-C-3	7/27/92	1 C var BF	0.218	0.178	0.692
51431	A-C-2	7/22/92	1 C var BF	0.2177	0.209	0.855
51432	A-C-4	7/22/92	1 C var NBF	0.098	0.119	0.4357
51433	A-C-5	7/22/92	1 C var BF	0.194	0.209	0.855
51434	A-S-1	7/27/92	2 C var NBF	0.227	0.178	0.692

51435	A-C-5	7/27/92	3 C var NBF	0.1472	0.44	0.543
51436	A-S-2	7/22/92	1 Mosq BF	0.189	0.18	0.78
51436B	A-S-2	7/22/92	2 Mosq BF	0.114	0.111	0.246
51437	A-S-1	7/29/92	1 Mosq BF	0.162	0.195	0.7515
51438	A-C-1	8/3/92	1 Mosq BF	0.187	0.195	0.7517
51439	A-C-2	7/22/92	1 Mosq BF	0.103	0.111	0.246
51439B	A-C-2	7/22/92	1 Mosq NBF	0.131	0.111	0.246
51440	A-S-3	7/29/92	1 C var NBF	0.047	0.09	0.216
51441	A-C-5	7/29/92	2 C var BF	0.091	0.111	0.246
51442	A-S-4	7/27/92	1 C var NBF	0.227	0.18	0.78
51443	A-S-3	8/3/92	2 C var NBF	0.192	0.18	0.78
51444	A-C-1	7/22/92	1 Mosq BF	0.103	0.111	0.246
51445	A-C-2	8/3/92	1 C crep BF	0.092	0.111	0.246
51446	A-S-5	7/27/92	2 C var NBF	0.176	0.195	0.7517
51447	A-C-3	7/27/92	1 Mangled NBF	0.16	0.18	0.78
51448	A-S-4	8/3/92	1 C haem NBF	0.187	0.18	0.78
51449	A-S-2	7/27/92	1 C crep NBF	0.188	0.18	0.78
51449B	A-S-2	7/27/92	1 C haem NBF	0.062	0.111	0.246
51450	A-C-5	7/20/92	1 Clear Wing	0.065	0.111	0.246
51451	A-S-3	7/22/92	1 Mangled NBF	0.062	0.111	0.246
51452	A-S-3	7/27/92	1 C crep BF	0.1632	0.18	0.78
51452B	A-S-3	7/27/92	1 C crep NBF	0.067	0.111	0.246
51453	A-S-2	7/22/92	2 C crep NBF	0.183	0.18	0.78
51454	A-C-5	8/3/92	1 C crep NBF	0.173	0.18	0.78
51454B	A-C-5	8/3/92	1 C haem NBF	0.087	0.111	0.246
51455	A-C-1	7/27/92	1 Mosq BF	0.159	0.18	0.78
51455B	A-C-1	7/27/92	1 Mosq NBF	0.206	0.18	0.78
51456	A-C-5	7/20/92	3 Mosq BF	0.229	0.18	0.78
51456B	A-C-5	7/20/92	4 Mosq NBF	0.169	0.195	0.7517
51457	A-C-2	8/3/92	7 Mosq BF	0.172	0.18	0.78
51457B	A-C-2	8/3/92	8 Mosq NBF	0.099	0.111	0.246
51458	A-C-5	8/3/92	4 Mosq BF	0.225	0.18	0.78
51458B	A-C-5	8/3/92	11 Mosq NBF	0.221	0.18	0.78
51459	A-S-2	8/3/92	3 Mosq BF	0.213	0.18	0.78
51459B	A-S-2	8/3/92	24 Mosq NBF	0.123	0.111	0.246
51460	A-S-3	7/12/92	1 Mosq BF	0.209	0.18	0.78
51460B	A-S-3	7/12/92	2 Mosq NBF	0.108	0.111	0.246
51461	A-S-5	8/3/92	3 Mosq BF	0.083	0.111	0.246
51461B	A-S-5	8/3/92	10 Mosq NBF	0.177	0.195	0.7517
51462	A-S-4	8/3/92	15 Mosq BF	0.104	0.119	0.4357
51462B	A-S-4	8/3/92	20 Mosq NBF	0.219	0.18	0.78
51463	A-C-4	8/3/92	14 Mosq BF	0.201	0.1952	0.7517
51463B	A-C-4	8/3/92	19 Mosq NBF	0.1255	0.122	0.4175
51464	A-S-3	7/29/92	1 C crep BF	0.105	0.111	0.246
51464B	A-S-3	7/29/92	1 C haem NBF	0.1285	0.122	0.4175
51465	A-S-1	7/22/92	2 C haem BF	0.155	0.18	0.78
51466	A-C-5	7/22/92	1 C crep NBF	0.1935	0.18	0.78
51467	A-C-3	7/29/92	1 C crep BF	0.1342	0.122	0.4175
51467B	A-C-3	7/29/92	1 C haem NBF	0.197	0.189	0.78
51468	A-S-1	7/27/92	2 A vexans BF	0.102	0.111	0.246

51468B	A-S-1	7/27/92	1 A vexans NBF	0.084	0.111	0.246
51468S	A-S-1	7/27/92	1 A dors BF	0.068	0.09	0.1435
51468Z	A-S-1	7/27/92	1 Aedes BF	0.046	0.053	0.1435
51469	A-S-5	7/22/92	1 Mosq BF	0.039	0.053	0.1435
51469B	A-S-5	7/22/92	5 Mosq NBF	0.0885	0.074	0.297
51470	A-S-4	7/22/92	4 Mosq BF	0.066	0.062	0.345
51470B	A-S-4	7/22/92	5 Mosq NBF	0.0437	0.053	0.1435
51471	A-S-2	7/27/92	3 Mosq BF	0.079	0.071	0.238
51471B	A-S-2	7/27/92	3 Mosq NBF	0.074	0.075	0.461
51472	A-C-2	7/29/92	1 Mosq BF	0.074	0.074	0.297
51472B	A-C-2	7/29/92	4 Mosq NBF	0.0953	0.074	0.297
51473	A-C-2	7/27/92	4 Mosq BF	0.067	0.071	0.238
51473B	A-C-2	7/27/92	6 Mosq NBF	0.165	0.0657	0.405
51474	A-C-3	7/22/92	6 Mosq BF	0.106	0.071	0.238
51474B	A-C-3	7/22/92	6 Mosq NBF	0.048	0.053	0.1435
51475	A-S-5	7/27/92	5 Mosq BF	0.086	0.071	0.238
51475B	A-S-5	7/27/92	2 Mosq NBF	0.0583	0.074	0.297
51476	A-C-5	7/27/92	4 Mosq BF	0.066	0.062	0.345
51476B	A-C-5	7/27/92	7 Mosq NBF	0.066	0.053	0.1435
51477	A-S-5	7/29/92	2 Mosq BF	0.066	0.071	0.238
51477B	A-S-5	7/29/92	7 Mosq NBF	0.044	0.053	0.1435
51478	A-S-3	7/29/92	6 Mosq BF	0.088	0.062	0.345
51478B	A-S-3	7/29/92	6 Mosq NBF	0.0585	0.053	0.1435
51479	A-S-2	7/29/92	3 Mosq BF	0.0417	0.053	0.1435
51479B	A-S-2	7/29/92	7 Mosq NBF	0.092	0.075	0.461
51480	A-S-4	7/27/92	2 Mosq BF	0.095	0.0657	0.405
51480B	A-S-4	7/27/92	10 Mosq NBF	0.532	0.16	10487
51481	A-S-4	7/29/92	4 Mosq BF	0.087	0.062	0.345
51481B	A-S-4	7/29/92	11 Mosq NBF	0.047	0.053	0.1435
51482	A-S-3	7/27/92	2 Mosq BF	0.08	0.0657	0.405
51482B	A-S-3	7/27/92	10 Mosq NBF	0.089	0.071	0.238
51483	A-C-5	7/22/92	9 Mosq BF	0.069	0.062	0.345
51483B	A-C-5	7/22/92	14 Mosq NBF	0.737	0.16	1.569
51484	A-S-2	8/3/92	1 C crep BF	0.053	0.052	0.171
51484B	A-S-2	8/3/92	1 C haem BF	0.0407	0.09	0.216
51485	A-C-1	7/22/92	3 Mangled	0.0445	0.053	0.1435
51486	A-C-3	8/3/92	2 C crep BF	0.0432	0.09	0.216
51486D	A-C-3	8/3/92	2 C haem BF	0.0472	0.09	0.216
51486E	A-C-3	8/3/92	1 C haem NBF	0.0442	0.053	0.1435
51487	A-C-4	8/3/92	1 C crep NBF	0.046	0.09	0.216
51487B	A-C-4	8/3/92	2 C crep BF	0.0417	0.053	0.1435
51487D	A-C-4	8/3/92	4 C haem NBF	0.093	0.111	0.246
51487E	A-C-4	8/3/92	2 C haem NBF	0.0407	0.09	0.216
51487Z	A-C-4	8/3/92	1 Mangled NBF	0.075	0.109	0.271
51488	A-S-3	8/3/92	2 C crep NBF	0.0427	0.09	0.216
51488B	A-S-3	8/3/92	3 C crep BF	0.0404	0.09	0.216
51489	A-S-1	7/27/92	1 C crep BF	0.045	0.052	0.171
51489B	A-S-1	7/27/92	1 C crep NBF	0.135	0.075	0.461
51489D	A-S-1	7/27/92	2 C haem NBF	0.0415	0.09	0.216
51490	A-S-3	8/3/92	1 Cul inornata BF	0.0647	0.053	0.1435

51490B	A-S-3	8/3/92	29 Mosq NBF	0.16	0.075	0.425
51490X	A-S-3	8/3/92	11 A vexans BF	0.074	0.075	0.461
51491	A-C-4	7/22/92	16 Mosq BF	0.282	0.185	0.937
51491B	A-C-4	7/22/92	27 Mosq NBF	0.0507	0.053	0.1435
51492	A-C-3	7/27/92	4 A vexans BF	0.042	0.053	0.1435
51492B	A-C-3	7/27/92	25 Mosq NBF	0.054	0.053	0.1435
51492T	A-C-3	7/27/92	1 C tars NBF	0.0422	0.09	0.216
51492N	A-C-3	7/27/92	1 A cinereus BF	0.1	0.111	0.246
51493	A-C-5	7/29/92	6 Mosq BF	0.076	0.075	0.425
51493B	A-C-5	7/29/92	18 Mosq NBF	0.198	0.075	0.425
51494	A-C-3	7/27/92	9 Mosq BF	0.09	0.0657	0.405
51494B	A-C-3	7/27/92	13 Mosq NBF	0.043	0.053	0.1435
51495	A-C-3	8/3/92	39 Mosq BF	0.05	0.053	0.1435
51495B	A-C-3	8/3/92	31 Mosq NBF	0.13	0.13	0.47
51496	A-C-4	7/27/92	2 Mosq BF	0.162	0.199	1.502
51496B	A-C-4	7/27/92	22 Mosq NBF	0.0537	0.053	0.1435
51496T	A-C-4	7/27/92	2 C tars BF	0.054	0.09	0.216
51496X	A-C-4	7/27/92	2 A vexans BF	0.0482	0.09	0.216
51497	A-C-4	7/29/92	16 Mosq BF	0.08	0.062	0.345
51497B	A-C-4	7/29/92	17 Mosq NBF	0.1405	0.074	0.297
51498	A-C-3	7/29/92	19 Mosq BF	0.072	0.0657	0.405
51498B	A-C-3	7/29/92	48 Mosq NBF	0.211	0.195	1.03
51499	F-C-2	8/17/92	2 C var BF	0.0567	0.09	0.216
51500	F-C-1	8/17/92	2 C var NBF	0.043	0.09	0.216
51501	F-C-3	8/17/92	20 C var NBF	0.046	0.053	0.1435
51502	F-C-4	8/17/92	1 C var BF	0.047	0.052	0.171
51503	F-C-2	8/17/92	3 C var NBF	0.333	0.185	0.937
51504	F-S-3	8/17/92	1 C var NBF	0.07	0.071	0.238
51505	F-C-4	8/17/92	5 C var NBF	0.053	0.052	0.171
51506	F-S-4	8/17/92	1 C var NBF	0.086	0.111	0.246
51507	F-C-1	8/17/92	7 C var NBF	0.098	0.109	0.271
51508	F-C-5	8/17/92	3 Mosq BF	0.069	0.075	0.461
51508B	F-C-5	8/17/92	16 Mosq NBF	0.354	0.195	1.03
51509	F-S-1	8/17/92	3 Mosq NBF	0.276	0.195	1.03
51509X	F-S-1	8/17/92	1 A vexans NBF	0.062	0.0657	0.405
51510	F-C-1	8/17/92	11 Mosq BF	0.0445	0.053	0.1435
51510B	F-C-1	8/17/92	1 Mosq NBF	0.055	0.058	0.226
51511	F-S-5	8/17/92	12 Mosq NBF	0.156	0.075	0.461
51512	F-C-2	8/17/92	18 Mosq BF	ND	ND	ND
51512B	F-C-2	8/17/92	3 Mosq NBF	0.246	0.146	1.176
51512T	F-C-2	8/17/92	1 C tars NBF	0.065	0.0657	0.405
51512P	F-C-2	8/17/92	8 C pipiens NBF	0.053	0.09	0.216
51513	F-S-4	8/17/92	3 Mosq BF	0.07	0.058	0.226
51513B	F-S-4	8/17/92	12 Mosq NBF	0.632	0.269	1.462
51514	F-C-3	8/17/92	23 Mosq BF	0.063	0.09	0.216
51514B	F-C-3	8/17/92	27 Mosq NBF	0.06525	0.09	0.216
51515	F-C-3	8/17/92	1 2-spot BF	0.058	0.071	0.238
51515B	F-C-3	8/17/92	1 C crep NBF	0.077	0.109	0.271
51516	F-C-5	8/17/92	1 C crep BF	0.48	0.185	0.937
51517	F-C-3	7/27/92	3 C var NBF	0.044	0.067	0.1595

51518	F-C-5	8/3/92	1 C var BF	0.0465	0.067	0.1595
51519	F-C-4	8/3/92	13 C var NBF	0.0435	0.067	0.1595
51520	F-C-3	7/27/92	6 C var BF	0.051	0.0456	0.177
51521	F-S-4	8/3/92	1 C var NBF	0.043	0.067	0.1595
51522	F-C-4	8/3/92	1 C var BF	0.044	0.067	0.1595
51523	F-C-5	8/3/92	2 C var NBF	0.04	0.067	0.1595
51524	F-C-1	7/27/92	2 C var NBF	0.051	0.0456	0.177
51525	F-C-4	7/27/92	3 C var BF	0.047	0.067	0.1595
51526	F-S-4	7/27/92	1 C var NBF	0.047	0.067	0.1595
51527	F-S-3	8/3/92	1 C var NBF	0.045	0.067	0.1595
51528	F-C-3	8/3/92	1 C haem BF	0.0402	0.0456	0.177
51529	F-C-2	7/27/92	2 C crep BF	0.044	0.067	0.1595
51530	F-S-4	8/3/92	2 C crep BF	0.0447	0.067	0.1595
51531	F-S-5	8/3/92	2 C var NBF	0.049	0.067	0.1595
51532	F-C-2	7/27/92	5 C var NBF	0.0405	0.0456	0.177
51533	F-C-1	7/27/92	2 C var BF	0.0385	0.0456	0.177
51534	F-C-2	7/27/92	5 C var BF	0.0402	0.0456	0.177
51535	F-C-5	7/27/92	3 C var NBF	0.04	0.0456	0.177
51536	F-S-1	8/3/92	1 C var NBF	0.054	0.0456	0.177
51537	F-C-5	7/27/92	2 C crep BF	0.0445	0.067	0.1595
51538	F-C-4	8/3/92	2 C haem BF	0.045	0.067	0.1595
51538C	F-C-4	8/3/92	1 C crep BF	0.0475	0.0456	0.177
51538D	F-C-4	8/3/92	1 C haem NBF	0.039	0.067	0.1595
51539	F-S-5	7/27/92	1 C var NBF	0.038	0.0456	0.177
51540	F-C-4	7/27/92	1 C haem NBF	0.0415	0.067	0.1595
51540C	F-C-4	7/27/92	1 C crep BF	0.044	0.067	0.159
51540Z	F-C-4	7/27/92	1 Mangled BF	0.0412	0.067	0.159
51541	F-S-3	8/3/92	1 C haem BF	0.046	0.0456	0.177
51542	F-S-5	8/3/92	2 C crep BF	0.0325	0.0456	0.177
51543	F-C-3	7/27/92	1 C crep BF	0.0427	0.067	0.1595
51544	F-C-4	7/27/92	1 C var NBF	0.038	0.0456	0.177
51545	F-C-3	8/3/92	3 C var NBF	0.035	0.0456	0.177
51546	F-C-2	8/3/92	1 C haem NBF	0.035	0.0456	0.177
51546D	F-C-2	8/3/92	1 C haem BF	0.0435	0.067	0.1595
51547	F-S-2	8/3/92	2 C crep BF	0.0405	0.0456	0.177
51548	F-C-5	8/3/92	3 C crep BF	0.0425	0.067	0.1595
51548D	F-C-5	8/3/92	2 C haem BF	0.0405	0.0456	0.177
51548Z	F-C-5	8/3/92	1 Mangled BF	0.0385	0.0456	0.177
51549	F-S-5	7/27/92	2 C crep BF	0.0447	0.0456	0.177
51549C	F-S-5	7/27/92	1 C crep NBF	0.0385	0.0456	0.177
51550	F-S-5	7/27/92	1 C tars NBF	0.044	0.0456	0.177
51551	F-C-1	8/12/92	8 C var BF	0.084	0.062	0.345
51552	F-S-5	8/12/92	1 C var NBF	0.056	0.058	0.226
51553	F-C-3	8/12/92	2 C var NBF	0.052	0.054	0.379
51554	F-C-5	8/12/92	1 C var NBF	0.051	0.058	0.226
51555	F-C-1	8/12/92	13 C var NBF	0.1	0.0657	0.405
51556	F-C-3	8/12/92	2 C var BF	0.058	0.058	0.226
51557	F-C-2	8/12/92	4 C var NBF	0.057	0.061	0.231
51558	F-C-1	8/12/92	1 Aedes BF	0.047	0.061	0.231
51559	F-S-5	8/12/92	1 C crep NBF	0.049	0.062	0.345

51559Z	F-S-5	8/12/92	1 Mangled NBF	0.531	0.0657	0.405
51560	F-C-5	9/10/92	8 Culex NBF	0.102	0.05	0.324
51561	F-S-5	8/12/92	11 Culex NBF	0.147	0.054	0.379
51562	F-C-3	9/17/92	2 Aedes BF	0.07	0.0657	0.405
51562B	F-C-3	9/17/92	1 Culex BF	0.1	0.0657	0.405
51562C	F-C-3	9/17/92	13 Aedes NBF	0.113	0.0642	0.238
51563	F-S-4	8/19/92	1 Aedes vex. NBF	0.061	0.05	0.324
51563B	F-S-4	8/19/92	1 C tars NBF	0.054	0.05	0.324
51563C	F-S-4	8/19/92	3 Aedes BF	0.094	0.0642	0.238
51563D	F-S-4	8/19/92	9 Culex NBF	0.052	0.06	0.159
51564	F-S-2	9/22/92	3 Culex NBF	0.119	0.054	0.379
51564B	F-S-2	9/22/92	1 C. tars NBF	0.061	0.06	0.159
51565	F-S-1	8/27/92	4 Aedes BF	0.079	0.05	0.324
51565B	F-S-1	8/27/92	4 Aedes NBF	0.0745	0.0642	0.238
51566	F-C-1	7/27/92	1 Mangled Mosq BF	0.055	0.054	0.379
51567	F-S-1	8/3/92	1 Aedes NBF	0.05	0.05	0.324
51568	F-S-4	8/27/92	4 Culex NBF	0.1057	0.06	0.159
51568B	F-S-4	8/27/92	1 Aedes NBF	0.052	0.06	0.159
51569	F-S-2	8/27/92	7 Culex NBF	0.1357	0.0642	0.238
51569B	F-S-2	8/27/92	6 Aedes NBF	0.063	0.0642	0.238
51569C	F-S-2	8/27/92	1 Aedes BF	0.052	0.054	0.379
51570	F-C-2	8/12/92	10 Aedes BF	0.073	0.0642	0.238
51570B	F-C-2	8/12/92	3 Aedes NBF	0.0767	0.05	0.324
51570C	F-C-2	8/12/92	13 Culex NBF	0.119	0.054	0.379
51571	F-S-3	8/19/92	19 Culex NBF	0.136	0.054	0.379
51571B	F-S-3	8/19/92	1 Aedes NBF	0.052	0.054	0.379
51571C	F-S-3	8/19/92	5 C tars NBF	0.054	0.05	0.324
51571D	F-S-3	8/19/92	1 Aedes BF	0.043	0.05	0.324
51571E	F-S-3	8/19/92	1 Culex BF	0.056	0.06	0.159
51571F	F-S-3	8/19/92	1 Culiseta NBF	0.068	0.05	0.324
51571G	F-S-3	8/19/92	1 Big Leg NBF	0.045	0.054	0.379
51573	F-C-4	8/12/92	8 Culex NBF	0.11	0.05	0.324
51573B	F-C-4	8/12/92	1 Aedes NBF	0.047	0.054	0.379
51573C	F-C-4	8/12/92	2 Aedes BF	0.053	0.05	0.324
51573D	F-C-4	8/12/92	4 C tars BF	0.056	0.05	0.324
51573E	F-C-4	8/12/92	4 C tars NBF	0.0418	0.074	0.297
51573F	F-C-4	8/12/92	1 Culiseta BF	0.209	0.075	0.425
51574	F-C-3	8/12/92	32 Culex NBF	0.113	0.06	0.159
51574B	F-C-3	8/12/92	1 Culex BF	0.079	0.054	0.379
51574C	F-C-3	8/12/92	1 Culiseta BF	0.1405	0.06	0.159
51574D	F-C-3	8/12/92	1 C tars BF	0.052	0.05	0.324
51574E	F-C-3	8/12/92	4 C tars NBF	0.057	0.054	0.379
51574F	F-C-3	8/12/92	1 Mangled Mosq NBF	0.054	0.0642	0.238
51574G	F-C-3	8/12/92	9 Aedes BF	0.063	0.06	0.159
51575	F-S-2	8/17/92	1 C crep BF	0.053	0.06	0.159
51575B	F-S-2	8/17/92	2 Mangled NBF	0.051	0.0642	0.238
51576	F-S-1	8/12/92	2 C cock NBF	0.053	0.06	0.159
51576B	F-S-1	8/12/92	1 C haem NBF	0.0505	0.06	0.159
51576C	F-S-1	8/12/92	1 2-spot NBF	0.052	0.0642	0.238
51577	F-S-1	8/17/92	3 2-spot NBF	0.048	0.0642	0.238

51577B	F-S-1	8/17/92	2 Mangled NBF	0.055	0.0642	0.238
51578	F-C-3	8/12/92	1 C crep BF	0.051	0.06	0.159
51578B	F-C-3	8/12/92	1 Mangled BF	0.05	0.0642	0.238
51579	F-S-5	8/12/92	4 C crep BF	0.05	0.06	0.159
51579B	F-S-5	8/12/92	2 C crep NBF	0.047	0.05	0.324
51580	F-C-1	8/12/92	8 Mangled	0.053	0.0642	0.238
51580B	F-C-1	8/12/92	1 2-spot BF	0.053	0.0642	0.238
51581	F-S-3	8/12/92	3 Mangled NBF	0.0487	0.06	0.159
51582	F-C-5	8/12/92	1 C crep BF	0.05	0.06	0.159
51582B	F-C-5	8/12/92	1 C crep NBF	0.052	0.06	0.159
51582C	F-C-5	8/12/92	1 C cock BF	0.047	0.05	0.324
51583	F-C-4	8/12/92	4 Mangled	0.053	0.071	0.238
51583B	F-C-4	8/12/92	3 C haem NBF	0.059	0.074	0.297
51584	F-C-1	8/17/92	2 C cock BF	0.049	0.0642	0.238
51584B	F-C-1	8/17/92	1 C cock NBF	0.049	0.05	0.324
51585	F-C-4	8/17/92	1 C crep BF	0.048	0.054	0.379
51585B	F-C-4	8/17/92	1 C crep NBF	0.061	0.06	0.159
51585C	F-C-4	8/17/92	1 Mangled NBF	0.051	0.054	0.379
51586	F-S-1	8/12/92	2 Aedes NBF	0.055	0.05	0.324
51587	F-S-2	8/12/92	1 C crep NBF	0.048	0.06	0.159
51588	F-C-2	8/12/92	5 Mangled	0.054	0.0642	0.238
51588B	F-C-2	8/12/92	1 C cock BF	0.047	0.054	0.379
51589	F-S-3	8/19/92	4 Mangled	0.0477	0.06	0.159
51589B	F-S-3	8/19/92	2 Clear Wing	0.061	0.075	0.425
51589C	F-S-3	8/19/92	3 C crep BF	0.055	0.071	0.238
51589D	F-S-3	8/19/92	3 C crep NBF	0.0538	0.074	0.297
51590	F-C-3	8/12/92	37 Culex NBF	0.128	0.054	0.379
51590B	F-C-3	8/12/92	13 Aedes BF	0.056	0.06	0.159
51590C	F-C-3	8/12/92	3 C tars NBF	0.072	0.0642	0.238
51590D	F-C-3	8/12/92	2 Aedes NBF	0.054	0.054	0.379
51590E	F-C-3	8/12/92	3 Culiseta BF	0.171	0.0642	0.238
51590F	F-C-3	8/12/92	2 Mangled Mosq NBF	0.057	0.05	0.324
51591	F-S-3	8/17/92	44 Culex NBF	0.111	0.05	0.324
51591B	F-S-3	8/17/92	9 C tars NBF	0.129	0.074	0.297
51591C	F-S-3	8/17/92	12 Aedes NBF	0.066	0.0642	0.238
51591D	F-S-3	8/17/92	2 Aedes BF	0.071	0.0642	0.238
51591E	F-S-3	8/17/92	3 Culiseta NBF	0.113	0.06	0.159
51592	F-C-5	8/12/92	35 Culex NBF	0.119	0.05	0.324
51592B	F-C-5	8/12/92	4 Aedes BF	0.056	0.0642	0.238
51592C	F-C-5	8/12/92	1 Culiseta BF	0.114	0.05	0.324
51592D	F-C-5	8/12/92	3 C tars NBF	0.057	0.054	0.379
51592E	F-C-5	8/12/92	1 C tars BF	0.051	0.054	0.379
51593	F-S-4	9/2/92	10 Culex NBF	0.623	0.16	1.487
51594	F-C-4	8/27/92	7 Culex NBF	0.584	0.16	1.569
51594B	F-C-4	8/27/92	4 Aedes BF	0.276	0.369	1.462
51594C	F-C-4	8/27/92	1 C tars NBF	0.114	0.195	1.572
51595	F-C-4	9/17/92	7 Aedes NBF	0.156	0.195	1.572
51595B	F-C-4	9/17/92	1 Culex NBF	0.294	0.16	1.487
51595C	F-C-4	9/17/92	1 Mangled Mosq NBF	0.247	0.16	1.569
51596	F-S-4	9/10/92	10 Aedes NBF	0.319	0.146	1.176

51596B	F-S-4	9/10/92	1 Culex NBF	0.369	0.187	1.48
51597	F-C-3	9/2/92	14 Aedes NBF	0.356	0.208	1.74
51597B	F-C-3	9/2/92	15 Culex NBF	0.317	0.185	0.937
51598	F-C-4	9/2/92	13 Aedes BF	0.18	0.195	1.03
51599	F-C-4	9/2/92	16 Culex NBF	0.348	0.195	1.03
51599B	F-C-4	9/2/92	5 Aedes NBF	0.424	0.16	1.569
51599C	F-C-4	9/2/92	1 Aedes BF	0.232	0.135	1.474
51600	F-C-3	9/17/92	1 C var BF	0.063	0.074	0.297
51601	F-C-5	9/10/92	1 C var NBF	0.0535	0.075	0.425
51602	F-C-3	9/17/92	1 C var NBF	0.0548	0.075	0.425
51603	F-C-3	9/22/92	4 C var BF	0.0735	0.075	0.425
51604	F-C-3	9/22/92	1 C var NBF	0.0591	0.075	0.461
51605	F-C-4	9/2/92	1 C var BF	0.074	0.075	0.461
51606	F-S-2	9/10/92	5 C var NBF	0.074	0.071	0.238
51607	F-S-4	9/10/92	4 C var NBF	0.065	0.075	0.461
51608	F-S-3	9/10/92	1 C var NBF	0.05	0.075	0.461
51609	F-C-4	9/2/92	3 C var NBF	0.066	0.075	0.461
51610	F-C-2	9/10/92	10 C var NBF	0.064	0.071	0.238
51611	F-S-5	9/17/92	1 C var NBF	0.11	0.075	0.425
51612	F-C-2	9/10/92	11 C var BF	0.0687	0.071	0.238
51613	F-C-1	9/22/92	13 C var NBF	0.063	0.075	0.425
51614	F-C-1	9/10/92	43 C var NBF	0.114	0.075	0.425
51615	F-C-3	9/2/92	1 C var BF	0.053	0.058	0.226
51616	F-S-3	8/19/92	3 C var NBF	0.054	0.071	0.238
51617	F-C-4	9/10/92	11 C var NBF	0.0845	0.075	0.461
51618	F-S-2	9/22/92	1 C var NBF	0.053	0.071	0.238
51619	F-C-5	8/19/92	2 C var BF	0.0713	0.074	0.297
51620	F-C-2	9/22/92	3 C var BF	0.0625	0.075	0.425
51621	F-S-1	9/22/92	1 C var NBF	0.062	0.071	0.238
51622	F-C-4	8/19/92	2 C var NBF	0.056	0.075	0.461
51623	F-C-4	8/19/92	1 C var BF	0.05	0.058	0.226
51624	F-C-4	9/17/92	7 C var NBF	0.087	0.071	0.238
51625	F-C-5	9/10/92	1 C var BF	0.05	0.075	0.425
51626	F-C-3	8/19/92	4 C var NBF	0.045	0.075	0.461
51627	F-C-2	9/17/92	2 C var BF	0.05	0.075	0.461
51628	F-C-2	9/17/92	16 C var NBF	0.0738	0.074	0.297
51629	F-S-5	8/19/92	3 C var NBF	0.052	0.075	0.461
51630	F-C-1	9/22/92	7 C var BF	0.052	0.071	0.238
51631	F-C-5	9/17/92	6 C var NBF	0.055	0.075	0.461
51632	F-C-1	8/27/92	2 C var BF	0.058	0.058	0.226
51633	F-C-2	8/27/92	2 C var BF	0.059	0.075	0.425
51634	F-C-1	8/27/92	2 C var NBF	0.06	0.058	0.226
51635	F-C-4	9/17/92	3 C var BF	0.058	0.075	0.461
51636	F-C-3	9/10/92	22 C var BF	0.091	0.058	0.226
51637	F-C-1	9/10/92	24 C var BF	0.08	0.075	0.461
51638	F-C-2	9/2/92	3 C var BF	0.07	0.075	0.425
51639	F-S-4	8/27/92	1 C var NBF	0.054	0.075	0.461
51640	F-C-2	9/2/92	10 C var NBF	0.0775	0.071	0.238
51641	F-C-3	9/10/92	11 C var NBF	0.0878	0.074	0.297
51642	F-S-3	9/2/92	1 C var NBF	0.055	0.071	0.238

51643	F-C-3	9/2/92	6 C var NBF	0.1133	0.074	0.297
51644	F-S-1	9/10/92	3 C var NBF	0.078	0.071	0.238
51645	F-S-4	8/19/92	4 C var NBF	0.057	0.058	0.226
51646	F-C-4	9/10/92	2 C var BF	0.061	0.075	0.461
51647	F-S-2	9/2/92	1 C var NBF	0.0565	0.074	0.297
51648	A-C-2	8/19/92	1 C var NBF	0.047	0.058	0.226
51649	A-S-4	8/19/92	1 C var NBF	0.06	0.185	0.937
51650	A-S-4	8/19/92	1 C var NBF	0.048	0.062	0.345
51651	A-C-3	8/19/92	2 C var BF	0.053	0.061	0.231
51652	A-C-4	8/19/92	1 C var BF	0.051	0.0581	0.226
51653	A-C-1	8/19/92	1 C var BF	0.048	0.061	0.231
51654	A-S-1	8/19/92	1 C var NBF	0.049	0.061	0.231
51655	A-C-3	8/19/92	1 C var BF	0.0515	0.062	0.345
51656	F-C-1	9/22/92	1 2-spot BF	0.0595	0.061	0.231
51657	F-S-1	9/10/92	1 2-spot BF	0.051	0.062	0.345
51658	F-S-3	9/2/92	1 Mangled	0.062	0.058	0.226
51659	A-S-2	8/19/92	1 C crep BF	0.058	0.0657	0.405
51660	A-S-4	8/19/92	1 Other	0.052	0.061	0.231
51661	F-S-5	9/17/92	1 C crep NBF	0.049	0.061	0.231
51662	F-C-2	9/10/92	1 Mangled BF	0.048	0.061	0.231
51663	F-S-1	8/19/92	2 C crep BF	0.056	0.058	0.226
51664	F-C-5	9/17/92	1 C crep NBF	0.048	0.061	0.231
51665	F-C-4	9/2/92	1 C crep BF	0.06	0.061	0.231
51666	F-C-5	8/27/92	1 C crep BF	0.067	0.0657	0.405
51667	F-S-2	9/22/92	1 C var NBF	0.0535	0.074	0.297
51668	F-S-5	9/10/92	1 C crep NBF	0.048	0.061	0.231
51669	F-S-5	9/2/92	1 C crep NBF	0.054	0.0657	0.405
51669B	F-S-5	9/2/92	1 C crep BF	0.05	0.062	0.345
51670	A-S-5	8/19/92	2 C crep BF	0.061	0.058	0.226
51671	F-S-3	9/10/92	1 C crep BF	0.055	0.058	0.226
51671Z	F-S-3	9/10/92	2 Mangled NBF	0.049	0.061	0.231
51672	F-S-2	9/10/92	1 C crep BF	0.056	0.0657	0.405
51672B	F-S-2	9/10/92	1 C crep NBF	0.052	0.058	0.226
51673	F-S-3	9/22/92	1 2-spot NBF	0.054	0.062	0.345
51674	A-C-4	8/19/92	2 C crep NBF	0.057	0.0657	0.405
51675	A-C-2	8/19/92	1 C crep BF	0.049	0.062	0.345
51675Z	A-C-2	8/19/92	2 Mangled NBF	0.052	0.061	0.231
51676	F-C-2	9/17/92	3 Mangled BF	0.056	0.0657	0.405
51677	F-C-5	8/19/92	1 C crep BF	0.049	0.058	0.226
51677Y	F-C-5	8/19/92	1 2-spot NBF	0.047	0.061	0.231
51678	F-C-5	9/10/92	2 C crep BF	0.06	0.0657	0.405
51678B	F-C-5	9/10/92	1 C crep NBF	0.046	0.054	0.379
51679	A-C-5	8/19/92	1 C pipiens NBF	0.074	0.075	0.425
51680	F-S-2	9/2/92	1 C pipiens NBF	0.052	0.075	0.425
51681	F-C-1	9/10/92	3 Mangled BF	0.0548	0.074	0.297
51682	F-C-1	8/27/92	1 C haem NBF	0.061	0.075	0.425
51682B	F-C-1	8/27/92	1 C crep NBF	0.049	0.075	0.425
51682Z	F-C-1	8/27/92	1 Mangled NBF	0.05	0.075	0.461
51683	F-C-4	8/19/92	2 C crep BF	0.05	0.061	0.231
51684	F-C-2	8/19/92	5 Mangled NBF	0.063	0.05	0.379

51684Y	F-C-2	8/19/92	7 2-spot BF	0.055	0.058	0.226
51685	A-S-3	8/19/92	2 C crep NBF	0.044	0.061	0.231
51685B	A-S-3	8/19/92	3 C crep BF	0.047	0.062	0.345
51686	F-C-3	9/10/92	1 C crep BF	0.051	0.062	0.345
51686Y	F-C-3	9/10/92	1 2-spot BF	0.049	0.061	0.231
51686Z	F-C-3	9/10/92	2 Mangled BF	0.051	0.061	0.231
51687	F-C-4	9/10/92	1 Mangled NBF	0.051	0.0657	0.405
51687B	F-C-4	9/10/92	1 C crep BF	0.051	0.062	0.345
51687C	F-C-4	9/10/92	1 C crep NBF	0.054	0.061	0.231
51689	F-S-2	8/19/92	2 C crep NBF	0.054	0.0657	0.405
51690	F-S-4	8/19/92	2 C crep NBF	0.0567	0.0657	0.405
51690Z	F-S-4	8/19/92	3 Mangled NBF	0.047	0.062	0.345
51691	F-S-5	8/19/92	2 C crep NBF	0.049	0.062	0.345
51691B	F-S-5	8/19/92	3 C crep BF	0.047	0.062	0.345
51692	A-C-3	8/19/92	3 C crep BF	0.049	0.061	0.231
51692B	A-C-3	8/19/92	3 C crep NBF	0.064	0.058	0.226
51692Z	A-C-3	8/19/92	1 Mangled BF	0.058	0.062	0.345
51693	F-C-4	9/17/92	21 Aedes BF	0.189	0.195	1.572
51693B	F-C-4	9/17/92	1 C tars BF	0.305	0.269	1.462
51694	F-C-2	9/2/92	9 Aedes NBF	0.504	0.135	1.474
51694B	F-C-2	9/2/92	1 Culex NBF	0.274	0.135	1.474
51694C	F-C-2	9/2/92	1 Mangled Mosq NBF	0.251	0.135	1.474
51695	F-S-3	9/2/92	10 Culex NBF	0.737	0.16	1.569
51695B	F-S-3	9/2/92	4 Aedes NBF	0.275	0.208	1.74
51695C	F-S-3	9/2/92	1 Culiseta NBF	0.412	0.135	1.474
51695D	F-S-3	9/2/92	1 C tars NBF	0.207	0.224	1.306
51696	F-S-3	8/12/92	13 Culex NBF	0.561	0.154	1.35
51696B	F-S-3	8/12/92	4 C tars NBF	0.182	0.165	1.47
51696C	F-S-3	8/12/92	2 Mangled Mosq NBF	0.071	0.13	0.47
51696D	F-S-3	8/12/92	5 Aedes NBF	0.26	0.146	1.176
51697	F-C-3	9/22/92	3 Aedes NBF	0.143	0.146	1.176
51697B	F-C-3	9/22/92	3 Culex NBF	0.502	0.154	1.35
51697C	F-C-3	9/22/92	1 Mangled Mosq NBF	0.344	0.269	1.462
51698	F-S-3	9/17/92	7 Aedes BF	0.276	0.224	1.306
51698B	F-S-3	9/17/92	1 Aedes NBF	0.229	0.208	1.74
51699	F-C-2	7/27/92	6 Aedes BF	0.375	0.146	1.176
51699B	F-C-2	7/27/92	1 C tars NBF	0.17	0.208	1.74
51699C	F-C-2	7/27/92	1 Culex NBF	0.122	0.195	1.03
51700	F-C-3	8/12/92	27 Culex NBF	0.272	0.185	0.937
51700B	F-C-3	8/12/92	19 Aedes BF	0.194	0.165	1.47
51700C	F-C-3	8/12/92	2 Culiseta BF	0.494	0.199	1.502
51700D	F-C-3	8/12/92	2 C tars NBF	0.38	0.165	1.47
51701	F-C-4	9/10/92	15 Aedes BF	0.208	0.195	1.572
51702	F-C-5	8/19/92	3 Aedes BF	0.172	0.199	1.502
51702B	F-C-5	8/19/92	5 Culex NBF	0.261	0.199	1.502
51702C	F-C-5	8/19/92	4 C tars NBF	0.192	0.199	1.502
51703	F-C-2	9/22/92	4 Culex NBF	0.461	0.195	1.572
51704	F-S-4	8/12/92	11 Culex NBF	0.408	0.199	1.502
51704B	F-S-4	8/12/92	1 Aedes NBF	0.136	0.195	1.572
51704C	F-S-4	8/12/92	1 Culiseta NBF	0.402	0.195	1.03

51704D	F-S-4	8/12/92	1 Culiseta BF	0.291	0.199	1.502
51704E	F-S-4	8/12/92	12 Mangled Mosq	0.428	0.165	1.47
51705	F-C-1	8/3/92	1 Aedes NBF	0.122	0.195	1.572
51706	F-C-3	9/22/92	1 Aedes BF	0.104	0.195	1.572
51707	F-S-4	9/2/92	1 Aedes BF	0.27	0.16	1.487
51708	F-S-4	9/17/92	2 Aedes BF	0.265	0.242	1.205
51709	F-S-4	9/10/92	2 Aedes BF	0.143	0.195	1.572
51710	A-S-1	8/19/92	2 Aedes BF	0.273	0.165	1.47
51711	F-C-5	9/17/92	1 Aedes NBF	0.209	0.195	1.572
51711B	F-C-5	9/17/92	1 Culex NBF	0.282	0.195	1.572
51712	F-S-5	9/10/92	1 Aedes NBF	0.128	0.195	1.572
51713	F-S-2	9/17/92	1 Aedes BF	0.256	0.165	1.47
51713B	F-S-2	9/17/92	1 Aedes NBF	0.208	0.165	1.47
51714	F-C-1	9/22/92	1 Aedes NBF	0.232	0.269	1.462
51715	F-S-1	9/22/92	1 Aedes NBF	0.353	0.269	1.462
51716	F-C-5	9/2/92	1 Aedes NBF	0.168	0.135	1.474
51717	F-S-2	8/19/92	1 Aedes BF	0.764	0.269	1.462
51717B	F-S-2	8/19/92	1 Culex NBF	0.31	0.165	1.47
51718	F-C-1	9/2/92	4 Aedes BF	0.371	0.146	1.176
51719	F-S-3	9/22/92	2 Culex NBF	0.338	0.16	1.567
51720	F-S-5	9/2/92	3 Aedes BF	0.134	0.199	1.502
51721	F-C-5	9/2/92	3 Aedes BF	0.181	0.165	1.47
51722	F-S-2	9/10/92	4 Aedes BF	0.275	0.154	1.35
51722B	F-S-2	9/10/92	2 Aedes NBF	0.202	0.199	1.502
51723	F-S-3	9/2/92	4 Aedes BF	0.181	0.165	1.47
51723B	F-S-3	9/2/92	1 Aedes NBF	0.124	0.195	1.572
51724	A-C-1	8/19/92	5 Aedes BF	0.112	0.165	1.47
51725	A-S-2	8/19/92	3 Aedes BF	0.287	0.195	1.03
51725B	A-S-2	8/19/92	2 C tars NBF	0.15	0.199	1.502
51726	F-S-1	9/10/92	3 Aedes BF	0.202	0.195	1.03
51726B	F-S-1	9/10/92	1 Culiseta BF	0.132	0.199	1.502
51727	F-C-5	9/10/92	4 Aedes BF	0.202	0.199	1.502
51728	F-S-5	8/19/92	5 Culex NBF	0.338	0.195	1.572
51728B	F-S-5	8/19/92	1 Aedes BF	0.091	0.13	0.47
51728C	F-S-5	8/19/92	1 C tars NBF	0.158	0.119	1.502
51729	F-S-5	9/2/92	7 Culex NBF	0.468	0.199	1.502
51730	A-C-2	8/19/92	5 Aedes BF	0.221	0.165	1.47
51730B	A-C-2	8/19/92	3 Culex NBF	0.167	0.199	1.502
51731	F-S-5	8/3/92	5 Aedes NBF	0.251	0.165	1.47
51731B	F-S-5	8/3/92	2 Culex NBF	0.514	0.165	1.47
51731C	F-S-5	8/3/92	2 C tars NBF	0.229	0.195	1.572
51732	F-C-5	9/17/92	5 Aedes BF	0.283	0.195	1.03
51733	F-C-5	8/27/92	5 Culex NBF	0.323	0.165	0.147
51733B	F-C-5	8/27/92	1 C tars NBF	0.269	0.165	1.47
51733C	F-C-5	8/27/92	1 Culiseta NBF	0.075	0.13	0.47
51734	A-S-5	8/19/92	4 Culex NBF	0.379	0.242	1.205
51734B	A-S-5	8/19/92	3 C tars NBF	0.204	0.199	1.502
51735	F-S-3	9/10/92	6 Aedes BF	0.201	0.165	1.47
51736	A-S-4	8/19/92	4 Culex NBF	0.258	0.195	1.03
51736B	A-S-4	8/19/92	2 C tars NBF	0.214	0.195	1.03

51736C	A-S-4	8/19/92	1 Aedes NBF	0.16	0.195	1.572
51736D	A-S-4	8/19/92	1 Aedes BF	0.216	0.195	1.03
51737	F-C-2	8/19/92	4 Aedes BF	0.148	0.199	1.502
51737B	F-C-2	8/19/92	3 Aedes NBF	0.299	0.195	1.03
51737C	F-C-2	8/19/92	1 Culex NBF	0.225	0.199	1.502
51738	F-S-3	8/27/92	15 Culex NBF	0.357	0.195	1.03
51738B	F-S-3	8/27/92	3 Culiseta NBF	0.248	0.195	1.572
51738C	F-S-3	8/27/92	2 Aedes BF	0.157	0.154	1.35
51738D	F-S-3	8/27/92	1 C tars NBF	0.269	0.154	1.35
51738E	F-S-3	8/27/92	3 Aedes NBF	0.247	0.135	1.474
51738F	F-S-3	8/27/92	2 Mangled Mosq NBF	0.365	0.187	1.48
51739	F-C-4	9/10/92	8 Aedes NBF	0.51	0.135	1.474
51739B	F-C-4	9/10/92	7 Culex NBF	0.463	0.195	1.03
51740	F-C-1	9/10/92	20 Aedes BF	0.435	0.16	1.569
51741	F-C-2	9/10/92	40 Aedes NBF	0.251	0.185	0.937
51741B	F-C-2	9/10/92	8 Aedes BF	0.317	0.224	1.306
51741C	F-C-2	9/10/92	2 Culex NBF	0.309	0.154	1.35
51742	F-S-3	8/12/92	37 Culex NBF	0.372	0.146	1.176
51742B	F-S-3	8/12/92	3 Culiseta NBF	0.615	0.224	1.306
51742C	F-S-3	8/12/92	2 Aedes BF	0.172	0.146	1.176
51742D	F-S-3	8/12/92	2 Aedes NBF	0.273	0.224	1.306
51742E	F-S-3	8/12/92	6 C tars NBF	0.226	0.146	1.176
51743	F-C-4	8/17/92	9 Aedes BF	0.097	0.16	1.569
51743B	F-C-4	8/17/92	54 Culex NBF	0.638	0.16	1.487
51743C	F-C-4	8/17/92	2 Culiseta BF	0.289	0.224	1.306
51743D	F-C-4	8/17/92	3 C tars NBF	0.175	0.173	1.25
51743E	F-C-4	8/17/92	5 C tars BF	0.146	0.154	1.35
51744	F-C-1	9/10/92	18 Aedes NBF	0.467	0.16	1.487
51744B	F-C-1	9/10/92	6 Aedes BF	0.496	0.16	1.569
51745	F-C-3	8/19/92	10 Aedes BF	0.333	0.208	1.74
51745B	F-C-3	8/19/92	12 Culex NBF	0.762	0.269	1.462
51745C	F-C-3	8/19/92	1 C tars NBF	0.181	0.195	1.03
51745D	F-C-3	8/19/92	1 C tars BF	0.118	0.195	1.572
51746	F-C-2	9/10/92	36 Aedes NBF	0.436	0.154	1.35
51746B	F-C-2	9/10/92	13 Aedes BF	0.229	0.208	1.74
51746C	F-C-2	9/10/92	1 Culex NBF	0.338	0.16	1.569
51747	F-C-4	8/19/92	31 Culex NBF	0.745	0.187	1.48
51747B	F-C-4	8/19/92	3 Culiseta NBF	0.367	0.146	1.176
51747C	F-C-4	8/19/92	3 Culiseta BF	0.301	0.187	1.48
51747D	F-C-4	8/19/92	4 Aedes BF	0.271	0.154	1.35
51747E	F-C-4	8/19/92	6 C tars BF	0.279	0.146	1.176
51747F	F-C-4	8/19/92	8 C tars NBF	0.244	0.135	1.474
51747G	F-C-4	8/19/92	2 Mangled Mosq NBF	0.199	0.16	1.487
51748	F-S-2	9/17/92	22 Aedes NBF	0.471	1.35	1.474
51749	A-S-3	8/19/92	15 Culex NBF	0.4355	0.16	1.487
51749B	A-S-3	8/19/92	5 Aedes BF	0.238	0.154	1.35
51749C	A-S-3	8/19/92	1 Aedes NBF	0.133	0.208	1.74
51749D	A-S-3	8/19/92	1 C tars NBF	0.123	0.146	1.176
51750	F-S-5	8/27/92	8 Aedes BF	0.25	0.187	1.48
51750B	F-S-5	8/27/92	9 Culex NBF	0.425	0.187	1.48

51750C	F-S-5	8/27/92	1 Culiseta BF	0.291	0.187	1.48
51751	F-C-2	9/17/92	14 Mangled Mosq BF	0.345	0.187	1.48
51751B	F-C-2	9/17/92	16 Aedes BF	0.438	0.187	1.48
51752	F-C-5	7/27/92	9 C tars NBF	0.293	0.16	1.487
51752B	F-C-5	7/27/92	4 C tars BF	0.332	0.29	1.462
51752C	F-C-5	7/27/92	3 Culex NBF	0.28	0.16	1.487
51752D	F-C-5	7/27/92	2 Aedes BF	0.11	0.185	0.937
51752E	F-C-5	7/27/92	1 Aedes NBF	0.237	0.224	1.306
51753	F-S-2	8/17/92	52 Culex NBF	0.605	0.135	1.474
51753B	F-S-2	8/17/92	16 Aedes NBF	0.222	0.199	1.502
51753C	F-S-2	8/17/92	1 Aedes BF	0.1455	0.165	1.47
51753D	F-S-2	8/17/92	6 C tars NBF	0.171	0.195	1.03
51753E	F-S-2	8/17/92	1 Culiseta NBF	0.179	0.195	1.572
51754	F-C-3	8/27/92	23 Aedes BF	0.143	0.195	1.03
51754B	F-C-3	8/27/92	9 Culex NBF	0.347	0.208	1.74
51754C	F-C-3	8/27/92	2 Aedes NBF	0.143	0.269	1.462
51754D	F-C-3	8/27/92	3 C tars NBF	0.179	0.242	1.205
51755	F-C-2	9/10/92	33 Aedes NBF	0.276	0.269	1.462
51755B	F-C-2	9/10/92	16 Aedes BF	0.27	0.208	1.74
51755C	F-C-2	9/10/92	1 Culex NBF	0.175	0.195	1.572
51756	F-C-2	9/10/92	37 Aedes NBF	0.303	0.199	1.502
51756B	F-C-2	9/10/92	9 Aedes BF	0.372	0.195	1.572
51756C	F-C-2	9/10/92	4 Culex NBF	0.266	0.242	1.205
51757	F-S-3	9/10/92	35 Aedes NBF	0.306	0.185	0.937
51757B	F-S-3	9/10/92	1 Aedes BF	0.114	0.185	0.937
51757C	F-S-3	9/10/92	3 Culex NBF	0.174	0.195	1.03
51757D	F-S-3	9/10/92	1 Culiseta NBF	0.211	0.199	1.502
51758	F-C-4	8/12/92	7 Aedes BF	0.165	0.16	1.487
51758B	F-C-4	8/12/92	37 Culex NBF	0.124	0.13	0.47
51758C	F-C-4	8/12/92	4 C tars NBF	0.203	0.165	1.47
51758D	F-C-4	8/12/92	1 C tars BF	0.045	0.16	1.569
51758E	F-C-4	8/12/92	1 Culiseta BF	0.19	0.195	1.03
51759	F-C-1	8/27/92	32 Aedes BF	0.172	0.154	1.35
51759B	F-C-1	8/27/92	6 Aedes NBF	0.215	0.195	1.03
51760	F-C-3	9/10/92	31 Aedes BF	0.172	0.269	1.462
51761	F-S-3	9/17/92	24 Aedes NBF	0.464	0.187	1.48
51761B	F-S-3	9/17/92	2 Culex NBF	0.354	0.224	1.306
51762	F-C-2	9/10/92	29 Aedes NBF	0.356	0.146	1.176
51762B	F-C-2	9/10/92	9 Aedes BF	0.203	0.135	1.474
51762C	F-C-2	9/10/92	1 Culex NBF	0.163	0.165	1.47
51763	A-C-4	8/19/92	5 Culex NBF	0.328	0.146	1.176
51763B	A-C-4	8/19/92	3 C tars NBF	0.192	0.16	1.487
51763C	A-C-4	8/19/92	3 Aedes BF	0.1535	0.269	1.462
51763D	A-C-4	8/19/92	1 Aedes NBF	0.371	0.208	1.74
51763E	A-C-4	8/19/92	1 Culiseta BF	0.25	0.146	1.176
51764	F-C-5	8/3/92	13 Aedes BF	0.128	0.135	1.474
51764B	F-C-5	8/3/92	3 Culiseta BF	0.59	0.16	1.569
51764C	F-C-5	8/3/92	2 Aedes NBF	0.241	0.16	1.569
51764D	F-C-5	8/3/92	10 C tars NBF	0.465	0.16	1.569
51764E	F-C-5	8/3/92	6 C tars BF	0.208	0.269	1.462

51764F	F-C-5	8/3/92	6 Culex NBF	0.847	0.269	1.462
51765	F-C-3	9/17/92	37 Aedes BF	0.192	0.224	1.306
51766	F-C-2	9/17/92	41 Aedes NBF	0.325	0.185	0.937
51766B	F-C-2	9/17/92	8 Aedes BF	0.273	0.16	1.569
51767	F-C-3	9/10/92	21 Culex NBF	0.302	0.185	0.937
51767B	F-C-3	9/10/92	6 Aedes BF	0.17	0.154	1.35
51767C	F-C-3	9/10/92	18 Aedes NBF	0.179	0.135	1.474
51768	F-C-2	9/10/92	49 Aedes BF	0.207	0.269	1.462
51769	F-C-2	9/17/92	50 Aedes BF	0.173	0.16	1.487
51770	F-C-3	9/2/92	120 Aedes BF	0.17	0.135	1.474
51771	F-C-2	9/2/92	50 Aedes BF	0.105	0.146	1.176
51772	F-C-3	9/10/92	50 Aedes BF	0.165	0.224	1.306
51773	A-C-3	8/19/92	22 Aedes BF	0.214	0.187	1.48
51773B	A-C-3	8/19/92	4 C tars NBF	0.261	0.16	1.569
51773C	A-C-3	8/19/92	4 Aedes NBF	0.331	0.185	0.937
51773D	A-C-3	8/19/92	18 Culex NBF	0.455	0.16	1.487
51773E	A-C-3	8/19/92	2 Culex BF	0.268	0.187	1.48
51774	F-C-1	9/10/92	50 Aedes BF	0.122	0.173	1.25
51775	F-C-2	9/10/92	50 Aedes BF	0.19	0.187	1.48
51776	F-C-2	9/17/92	50 Aedes BF	0.1545	0.16	1.487
51777	F-C-1	9/10/92	50 Aedes BF	0.133	0.16	1.487
51778	F-C-3	9/17/92	50 Aedes BF	0.202	0.16	1.569
51779	F-C_2	9/17/92	50 Aedes BF	0.136	0.173	1.25
51780	F-C-1	9/10/92	50 Aedes BF	0.172	0.208	1.74
51781	F-C-1	9/10/92	50 Aedes BF	0.144	0.154	1.35
51782	F-C-2	9/10/92	50 Aedes BF	0.188	0.224	1.306
51783	F-C-2	9/10/92	50 Aedes BF	0.198	0.135	1.474
51784	F-C-2	9/10/92	50 Aedes BF	0.212	0.224	1.306
51785	F-C-3	8/3/92	21 Aedes BF	0.181	0.16	1.487
51785B	F-C-3	8/3/92	4 C tars NBF	0.202	0.16	1.569
51785C	F-C-3	8/3/92	5 Culex NBF	0.488	0.16	1.569
51785D	F-C-3	8/3/92	2 Aedes NBF	0.264	0.187	1.48
51785E	F-C-3	8/3/92	2 Culiseta BF	0.577	0.208	1.74
51786	F-S-2	8/3/92	34 Aedes NBF	0.271	0.269	1.462
51786B	F-S-2	8/3/92	4 C tars NBF	0.176	0.135	1.474
51786C	F-S-2	8/3/92	1 Aedes BF	0.133	0.173	1.25
51786D	F-S-2	8/3/92	5 Culex NBF	0.313	0.224	1.306
51787	F-C-3	8/17/92	17 Aedes BF	0.326	0.173	1.28
51787B	F-C-3	8/17/92	20 Culex NBF	0.693	0.16	1.487
51787C	F-C-3	8/17/92	4 Aedes NBF	0.236	0.269	1.462
51787D	F-C-3	8/17/92	2 Culex BF	0.117	0.135	1.474
51787E	F-C-3	8/17/92	8 C tars NBF	0.375	0.269	1.462
51788	F-C-3	8/19/92	11 Mangled	0.361	0.173	1.25
51789	F-C-2	9/10/92	50 Aedes BF	0.301	0.187	1.48
51790	F-C-3	7/27/92	21 Aedes BF	0.297	0.269	1.462
51790B	F-C-3	7/27/92	2 Culiseta BF	0.315	0.154	1.35
51790C	F-C-3	7/27/92	5 Culex NBF	0.313	0.16	1.487
51790D	F-C-3	7/27/92	6 Aedes NBF	0.215	0.154	1.35
51790E	F-C-3	7/27/92	2 C tars BF	0.241	0.16	1.569
51790F	F-C-3	7/27/92	5 C tars NBF	0.311	0.208	1.74

51791	F-C-3	9/10/92	50 Aedes BF	0.146	0.154	1.35
51792	F-S-2	9/10/92	40 Aedes NBF	0.297	0.185	0.937
51792B	F-S-2	9/10/92	3 Aedes BF	0.317	0.146	1.176
51792C	F-S-2	9/10/92	7 Culex NBF	0.351	0.185	0.937
51793	F-S-4	8/3/92	20 Aedes NBF	0.502	0.187	1.48
51793B	F-S-4	8/3/92	2 Aedes BF	0.159	0.154	1.35
51793C	F-S-4	8/3/92	5 C tars NBF	0.443	0.16	1.569
51793D	F-S-4	8/3/92	5 Culex NBF	0.353	0.135	1.474
51793E	F-S-4	8/3/92	4 Culiseta NBF	0.407	0.208	1.74
51794	A-C-2	8/17/92	23 Aedes BF	ND	ND	ND
51794B	A-C-2	8/17/92	23 Culex NBF	0.4	0.208	1.74
51794C	A-C-2	8/17/92	3 Aedes NBF	0.132	0.173	1.25
51794D	A-C-2	8/17/92	1 C tars NBF	0.191	0.16	1.487
51795	F-S-2	8/17/92	43 Culex NBF	0.204	0.146	1.176
51795B	F-S-2	8/17/92	3 C tars NBF	0.182	0.173	1.25
51795C	F-S-2	8/17/92	4 Aedes NBF	0.15	0.146	1.176
51796	F-C-1	9/10/92	40 Aedes NBF	0.344	0.173	1.25
51796B	F-C-1	9/10/92	10 Aedes BF	0.26	0.208	1.74
51797	F-C-3	7/27/92	11 Aedes BF	ND	ND	ND
51797B	F-C-3	7/27/92	13 C tars NBF	0.338	0.224	1.306
51797C	F-C-3	7/27/92	5 Aedes NBF	0.361	0.224	1.306
51797D	F-C-3	7/27/92	1 Culiseta BF	0.31	0.224	1.306
51797E	F-C-3	7/27/92	7 Culex NBF	0.4155	0.269	1.462
51798	F-S-2	9/10/92	48 Aedes NBF	0.284	0.208	1.74
51798B	F-S-2	9/10/92	1 Culex NBF	0.133	0.146	1.176
51799	F-C-3	7/27/92	19 Aedes BF	0.241	0.154	1.35
51799B	F-C-3	7/27/92	11 Culex NBF	0.369	0.187	1.48
51799C	F-C-3	7/27/92	6 C tars NBF	0.325	0.154	1.35
51799D	F-C_3	7/27/92	4 Aedes NBF	0.344	0.187	1.48
51800	F-S-2	8/12/92	51 Mosq	0.602	0.224	1.306
51801	F-S-2	9/10/92	50 Mosq	0.239	0.146	1.176
51802	F-C-2	8/12/92	50 Mosq	0.463	0.224	1.306
51803	F-C-2	8/12/92	50 Mosq	0.422	0.135	1.474
51804	F-C-3	8/27/92	50 Mosq	0.302	0.224	1.306
51805	F-C-3	8/27/92	50 Mosq	ND	ND	ND
51806	F-C-3	8/19/92	50 Mosq	0.253	0.135	1.474
51807	F-S-1	9/10/92	52 Mosq	ND	ND	ND
51808	F-C-5	8/19/92	50 Mosq	0.279	0.173	1.25
51809	F-C-3	8/17/92	83 Mosq	0.372	0.185	0.937
51810	F-C-2	8/3/92	65 Mosq	0.229	0.16	1.487
51811	F-C-2	8/3/92	71 Mosq	0.234	0.224	1.306
51812	F-S-1	8/3/92	60 Mosq	0.415	0.187	1.48
51813	F-C-4	7/27/92	60 Mosq	0.213	0.185	0.937
51814	F-C-2	8/3/92	60 Mosq	0.195	0.173	1.25
51815	F-C-4	8/3/92	60 Mosq	ND	ND	ND
51816	F-S-2	9/10/92	50 Mosq	0.458	0.154	1.35
51817	F-C-2	8/27/92	53 Mosq	0.263	0.208	1.74
51818	F-S-2	8/10/92	50 Mosq	0.4	0.208	1.74
51819	F-C-3	7/27/92	45 Mosq	0.211	0.185	0.937
51820	F-C-3	8/19/92	50 Mosq	0.272	0.16	1.569

51821	F-C-2	8/3/92	50 Mosq	0.389	0.187	1.48
51822	F-C-4	8/3/92	55 Mosq	0.239	0.154	1.35
51823	F-C-2	8/27/92	50 Mosq	0.326	0.208	0.174
51824	F-S-3	8/31/92	52 Mosq	0.233	0.208	1.74

Acc# = Accession number of pool

ND = Not Done

BF = Blood-fed

NBF = Non-blood-fed

F = Fields

A = Ausman

C = Calf

S = Sheep

Mangled = Unable to identify further

Culex = Culex species

Aedes = Aedes species

Culiseta = Culiseta species

Mosq = Mosquitoes

C var = Culicoides variipennis

C haem = Culicoides haematopodus

C crep = Culicoides crepuscularis

C other = Culicoides other species

C 2-spot = Culicoides 2-spot winged sp.

C clear wing = Culicoides clear winged sp.

C pipiens = Culex pipiens

C tars = Culex tarsalis

A vexans = Aedes vexans

APPENDIX C

Examples of larval pools and corresponding ELISA values

Acc #	Pool site	Date	Sample	<u>ELISA Values</u>	
				<u>Neg. Con</u>	<u>Pos. Con</u>
				<u>+ 3 SD</u>	
11049	C1	7/11/91	0.2733	0.3376	0.6386
11051	C3	7/11/91	0.2908	0.3376	0.6386
11052	C4	7/11/91	0.267	0.371	0.73
11053	C5	7/11/91	0.2936	0.3038	0.947
11054	AA1	6/20/91	0.261	0.371	0.73
11056	BB1	7/11/91	0.3121	0.3984	0.5833
11057	BB2	7/11/91	0.264	0.371	0.73
11059	BB4	7/11/91	0.247	0.371	0.73
11071	C6	7/11/91	0.403	0.528	0.746
11072	C7	7/11/91	0.363	0.528	0.746
11073	CC1	7/11/91	0.358	0.528	0.746
11074	CC2	7/11/91	0.369	0.528	0.746
11075	R1	7/11/91	0.368	0.528	0.746
11076	R2	7/11/91	0.346	0.528	0.746
11077	R3	7/11/91	0.358	0.528	0.746
11078	R4	7/11/91	0.376	0.528	0.746
11079	R5	7/11/91	0.353	0.528	0.746
11080	R1	7/11/91	0.342	0.528	0.746
11081	R6	7/11/91	0.348	0.528	0.746
11082	E1	7/11/91	0.358	0.528	0.746
11084	E3	7/11/91	0.359	0.528	0.746
11085	E4	7/11/91	0.368	0.47	0.631
11086	D1	7/11/91	0.376	0.47	0.631
11087	D2	7/11/91	0.355	0.47	0.631
11088	D3	7/11/91	0.347	0.47	0.631
11091	V1	6/26/91	0.2901	0.3984	0.5833
11093	V3	6/26/91	0.2631	0.3376	0.6386
11094	V4	6/26/91	0.336	0.47	0.631
11095	V5	6/26/91	0.3015	0.3038	0.947
11096	V6	6/26/91	0.32	0.47	0.631
11098	V8	6/26/91	0.331	0.47	0.631
11099	V9	6/26/91	0.32	0.47	0.631
11100	V10	6/26/91	0.287	0.3376	0.7386
11102	V12	6/26/91	0.348	0.47	0.631
11103	V13	6/26/91	0.292	0.3038	0.947
11104	V14	6/26/91	0.333	0.47	0.631

11110	B2	7/11/91	0.318	0.47	0.631
11131	N1	6/20/91	0.326	0.47	0.631
11132	P1	6/26/91	0.311	0.47	0.631
11167	AA1	6/5/91	0.075	0.092	0.204
11168	AA2	6/5/91	0.082	0.092	0.204
11169	AA3	6/5/91	0.083	0.092	0.204
11170	AA4	6/5/91	0.075	0.092	0.204
11171	AA5	6/5/91	0.075	0.092	0.204
11172	AA6	6/5/91	0.082	0.092	0.204
11173	AA7	6/5/91	0.078	0.092	0.204
11220	C13	6/5/91	0.075	0.087	0.182
11221	C14	6/5/91	0.287	0.388	1.061
11223	I2	6/11/91	0.0962	0.176	1.3225
11224	I3	6/11/91	0.087	0.101	0.199
11225	I4	6/11/91	0.288	0.388	1.061
11226	I5	6/11/91	0.2405	0.4649	1.182
11227	L1	6/11/91	0.2493	0.4649	1.182
11228	X1	6/11/91	0.2815	0.388	1.061
11230	W1	6/11/91	0.3136	0.388	1.061
11231	K1	6/11/91	0.2415	0.4649	1.182
11232	J1	6/11/91	0.0772	0.176	1.3225
11234	Y2	6/11/91	0.2338	0.4649	1.182
11235	Y3	6/11/91	0.414	0.577	1.367
11236	Y4	6/11/91	0.2466	0.4649	1.182
11237	Y5	6/11/91	0.3146	0.3376	0.6386
11238	Y6	6/11/91	0.2471	0.4649	1.182
11239	Y7	6/11/91	0.2398	0.4649	1.182
11240	Y8	6/11/91	0.4	0.577	1.367
11241	C2	6/20/91	0.086	0.101	0.199
11242	C3	6/20/91	0.082	0.101	0.199
11243	C4	6/20/91	0.081	0.101	0.199
11244	A1	6/20/91	0.086	0.101	0.199
11245	E1	6/20/91	0.082	0.101	0.199
11246	C1	6/20/91	0.085	0.101	0.199
11247	D1	6/20/91	0.3085	0.3338	0.947
11248	F1	6/20/91	0.087	0.101	0.199
11249	F2	6/20/91	0.084	0.101	0.199
11250	F3	6/20/91	0.084	0.101	0.199
11251	M1	6/20/91	0.09	0.101	0.199
11252	Q1	6/20/91	0.085	0.101	0.199
11253	Q2	6/20/91	0.085	0.101	0.199
11254	Q3	6/20/91	0.2885	0.3038	0.947
11255	Q4	6/20/91	0.086	0.101	0.199
11256	Q5	6/20/91	0.084	0.101	0.199
11257	Q6	6/20/91	0.089	0.101	0.199
11259	O2	6/20/91	0.308	0.3984	0.5833
11266	BB1	6/20/91	0.283	0.3038	0.947

11268	CC1	6/20/91	0.284	0.3376	0.6386
11297	BB6	6/5/91	0.078	0.092	0.204
11298	BB7	6/5/91	0.075	0.087	0.182
11300	BB9	6/5/91	0.072	0.087	0.182
11301	BB10	6/5/91	0.077	0.087	0.182
11303	BB12	6/5/91	0.073	0.087	0.182
11304	BB13	6/5/91	0.074	0.087	0.182
11305	BB14	6/5/91	0.084	0.092	0.204
11306	BB15	6/5/91	0.072	0.087	0.182
11308	BB17	6/5/91	0.084	0.092	0.204
11309	BB18	6/5/91	0.078	0.087	0.182
11310	BB19	6/5/91	0.077	0.087	0.182
11311	BB20	6/5/91	0.082	0.092	0.204
11312	CC1	6/5/91	0.074	0.087	0.182
11313	CC2	6/5/91	0.079	0.092	0.204
11314	CC3	6/5/91	0.072	0.087	0.182
11315	CC4	6/5/91	0.071	0.087	0.182
11316	CC5	6/5/91	0.064	0.087	0.182
11317	CC6	6/5/91	0.078	0.092	0.204
11318	CC7	6/5/91	0.085	0.092	0.204
11319	CC8	6/5/91	0.072	0.087	0.182
11320	CC9	6/5/91	0.085	0.092	0.204
11321	CC10	6/5/91	0.079	0.092	0.204
11335	Y1	8/14/91	0.39	0.389	1.051
11336	Y2	8/14/91	0.376	0.389	1.051
11337	H1	8/14/91	0.378	0.389	1.051
11338	G1	8/14/91	0.351	0.389	1.051
11339	K1	8/14/91	0.369	0.389	1.051
11340	K2	8/14/91	0.361	0.389	1.051
11341	K3	8/14/91	0.359	0.389	1.051
11342	K4	8/14/91	0.367	0.389	1.051
11343	T1	8/14/91	0.342	0.389	1.051
11344	T2	8/14/91	0.314	0.389	1.051
11345	J1	8/14/91	0.325	0.389	1.051
11346	L1	8/14/91	0.316	0.389	1.051
11347	I1	8/14/91	0.33	0.389	1.051
11348	I2	8/14/91	0.313	0.389	1.051
11349	I3	8/14/91	0.309	0.389	1.051
11350	I4	8/14/91	0.33	0.389	1.051
11351	I1	7/19/91	0.426	0.495	1.257
11352	V2	7/19/91	0.397	0.495	1.257
11353	L1	7/19/91	0.383	0.495	1.257
11354	V5	7/19/91	0.387	0.495	1.257
11355	K1	7/19/91	0.374	0.495	1.257
11356	G1	7/19/91	0.381	0.495	1.257
11357	W1	7/19/91	0.38	0.495	1.257
11358	K3	7/19/91	0.378	0.495	1.257

11359	V8	7/19/91	0.362	0.495	1.257
11360	T1	7/19/91	0.353	0.495	1.257
11361	T2	7/19/91	0.327	0.495	1.257
11362	V9	7/19/91	0.331	0.495	1.257
11363	V10	7/19/91	0.367	0.495	1.257
11364	V3	7/19/91	0.331	0.495	1.257
11365	J1	7/19/91	0.33	0.495	1.257
11366	V7	7/19/91	0.326	0.495	1.257
11367	V1	7/19/91	0.427	0.577	1.367
11368	K1	7/19/91	0.383	0.577	1.367
11369	V4	7/19/91	0.401	0.577	1.367
11370	K2	7/19/91	0.386	0.577	1.367
11371	V6	7/19/91	0.396	0.577	1.367
11372	F2	7/11/91	0.405	0.577	1.367
11373	X1	6/25/91	0.412	0.577	1.367
11374	X1	8/14/91	0.398	0.577	1.367
11375	V1	8/14/91	0.396	0.577	1.367
11376	W1	8/14/91	0.406	0.577	1.367
11377	F1	7/11/91	0.395	0.577	1.367
11378	D1	7/26/91	0.385	0.577	1.367
11379	I1	6/26/91	0.396	0.577	1.367
11380	E2	8/23/91	0.411	0.577	1.367
11381	C1	8/23/91	0.2766	0.3984	0.5833
11382	C2	8/23/91	0.3145	0.3984	0.5833
11383	BB1	8/23/91	0.3171	0.3984	0.5833
11384	N1	8/23/91	0.3308	0.3984	0.5833
11385	CC1	8/23/91	0.2451	0.3038	0.947
11386	P1	8/23/91	0.3155	0.3984	0.5833
11387	P2	8/23/91	0.3565	0.3984	0.5833
11403	G1	8/30/91	0.379	0.3984	0.5833
11404	G2	8/30/91	0.2958	0.3038	0.947
11405	G3	8/30/91	0.3128	0.3376	0.6386