TEMPERATURE AND THE TRANSMISSION OF ARBOVIRUSES BY *CULICOIDES* BITING MIDGES

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ABSTRACT

Culicoides biting midges are economically important as vectors of several arboviruses of domestic and wild animals. The most important of these viruses are bluetongue virus (BTV), which infects ruminants, and African horse sickness virus (AHSV), which infects equids. Climatic factors can affect the capacity of *Culicoides* to transmit these viruses by influencing the size of adult *Culicoides* populations and the proportion of adults within a population capable of transmitting the viruses. Here, I report the results of a series of studies investigating the influence of one such factor, temperature, on virus transmission. The optimum temperatures for recruitment of adult midges from the immature stages were estimated to be 25-30°C for *C. nubeculosus* and 25-35°C for *C. variipennis sonorensis*, while the minimum temperatures for development were 8.1°C and 10.7°C respectively. The proportion of adult *C. variipennis sonorensis* capable of transmitting BTV and AHSV, as well as epizootic haemorrhagic disease virus (EHDV) was greatest at 27-30°C. Thus although longevity of adult *C. variipennis sonorensis* was reduced at these high temperatures, this was more than compensated for by the accompanying decrease in the duration of the viral extrinsic incubation period (EIP). In contrast, at cooler temperatures (15-17°C) adult longevity was extended, but the EIP was disproportionately prolonged meaning that few adult midges were capable of virus transmission. The impact of temperature on the vector competence of *Culicoides* vector populations (i.e. proportion of midges with virus susceptible genotypes that develop susceptible phenotypes) varied with the virus species and serotype. Vector competence increased with temperature for *C. variipennis sonorensis* infected with AHSV4 or EHDV1, whereas temperature had no effect on vector competence of *C. variipennis sonorensis* infected with BTV10 or BTV16 and *C. imicola* infected with AHSV8. In addition, I found that exposure of immature *C. nubeculosus* (a non-vector species) to temperatures close to their upper lethal limit (33°C) could induce vector competence for BTV and AHSV. The distribution of *Culicoides* species is also influenced by climate and here I show that the range of *C. imicola* (principally an Afro-Asian species) in Europe is limited by temperature. The information from these studies can be used to assess how global warming will affect the distribution and seasonal occurrence of BTV and AHSV.

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Thanks also go to my family, although on several occasions they have suggested that counting insects is a rather unusual career choice. I am also indebted to Mark Fellowes who has supported me during the last three years and amazingly, endured the (large number of) writing-up months.

DECLARATION

I declare that the work in this dissertation was carried out in accordance with the Regulations of the University of Bristol. The work is original except where indicated by special reference in the text and no part of the dissertation has been submitted for any other degree.

Any views expressed in the dissertation are those of the author and in no way represent those of the University of Bristol.

The dissertation has not been presented to any other University for examination either in the United Kingdom or overseas.

Emma Wittmann.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: GENERAL INTRODUCTION[1]

1.1 INTRODUCTION

Climate change is one of the most serious environmental issues of our day. In the last century the global mean temperature rose by 0.5°C (Jones and Wigley, 1990) and if no steps are taken to limit greenhouse gas emissions, temperatures could rise by a further 2°C by 2100 (Intergovernmental Panel on Climate Change, 1996; Houghton, 1997). This predicted rate of change is greater than global temperatures have changed at any time over the past ten thousand years. In addition, changes in precipitation, wind patterns and climate variability are likely (Houghton, 1997). The impact of these changes will be enormous.

One of the most immediate and noticeable impacts of climate change will be an alteration in the distribution and abundance of insect species (Sutherst, 1990). This is particularly worrying in the case of insects that transmit pathogens or parasites to humans and livestock, since it is also likely to affect the prevalence of insect-borne diseases.

Culicoides biting midges (Diptera: Ceratopogonidae) are economically important as vectors of several arboviruses of domestic and wild animals, including bluetongue virus (BTV), which infects ruminants and African horse sickness virus (AHSV), which infects equids. Indeed, the diseases caused by these viruses, bluetongue (BT) and African horse sickness (AHS), are of such major international concern that they have attained Office International des Epizooties (OIE) list 'A' status. That is, diseases which have the potential to spread rapidly from one country to another, to cause high mortality and morbidity in susceptible animals, and to affect international trade in livestock and livestock products. World-wide it has been estimated that BTV results in losses of $3 billion/year (Tabachnick *et al.* 1996). The effects of AHSV are also devastating and during the most recent outbreak of AHSV in Iberia and Morocco (1987-1991) 2000 horses died and more than 350,000 had to be vaccinated (Rodriguez *et al.*, 1992; Mellor 1993).

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To assess the potential impact of climate change on *Culicoides*-transmitted viruses, particularly with regards to Europe, it is essential first to understand how climatic factors affect the vector and the viruses. Here I begin with an overview of *Culicoides* biology, BTV and AHSV, and the capacity of *Culicoides* to transmit these viruses. I then describe what is currently known about the influence of climate and weather on *Culicoides* and virogenesis and highlight the areas where further information is required if we are to gain insight into the potential influence of climate change on the viral diseases. Finally, I describe the aims of this thesis.

1.2 *CULICOIDES* BITING MIDGES

Culicoides biting midges (Figure 1.1) are among the world's smallest haematophagous flies, ranging in size from 1-3 mm (Meiswinkel *et al.*, 1994). They occur on all inhabited landmasses, except for New Zealand, the Hawaiian Islands and Patagonia (Boorman, 1993; Meiswinkel *et al.*, 1994) and there are at least 1210 different species (Borkent and Wirth, 1997).

Life history

The life cycle consists of egg, four larval instars, pupa and adult stages. The immatures require moisture and organic matter for development and breeding sites include damp or saturated soils, bogs, marshes, swamps, tree holes, animal dung and rotting fruits or other vegetation (Meiswinkel *et al.*, 1994; Mellor, 1996). The duration of the life cycle depends on the species and climatic conditions, varying from 7 days in the tropics to 7 months in temperate regions, where most species diapause as fourth instar larvae during winter (Braverman, 1994). The life-span of the adults is usually short and also depends on the ambient conditions. Most adults survive less than 20 days, although occasionally they live for up to 90 days (Mellor *et al.*, 2000).

Females feed on blood, which provides protein for the development of eggs and one blood-meal is usually required for each batch of eggs matured. The frequency of feeding is therefore linked to the rate of egg development, which is itself dependent on the species and the ambient temperature. Many species blood-feed on a range of hosts, for example, *C. imicola* will feed on cattle, sheep, horses, pigs and birds (Braverman

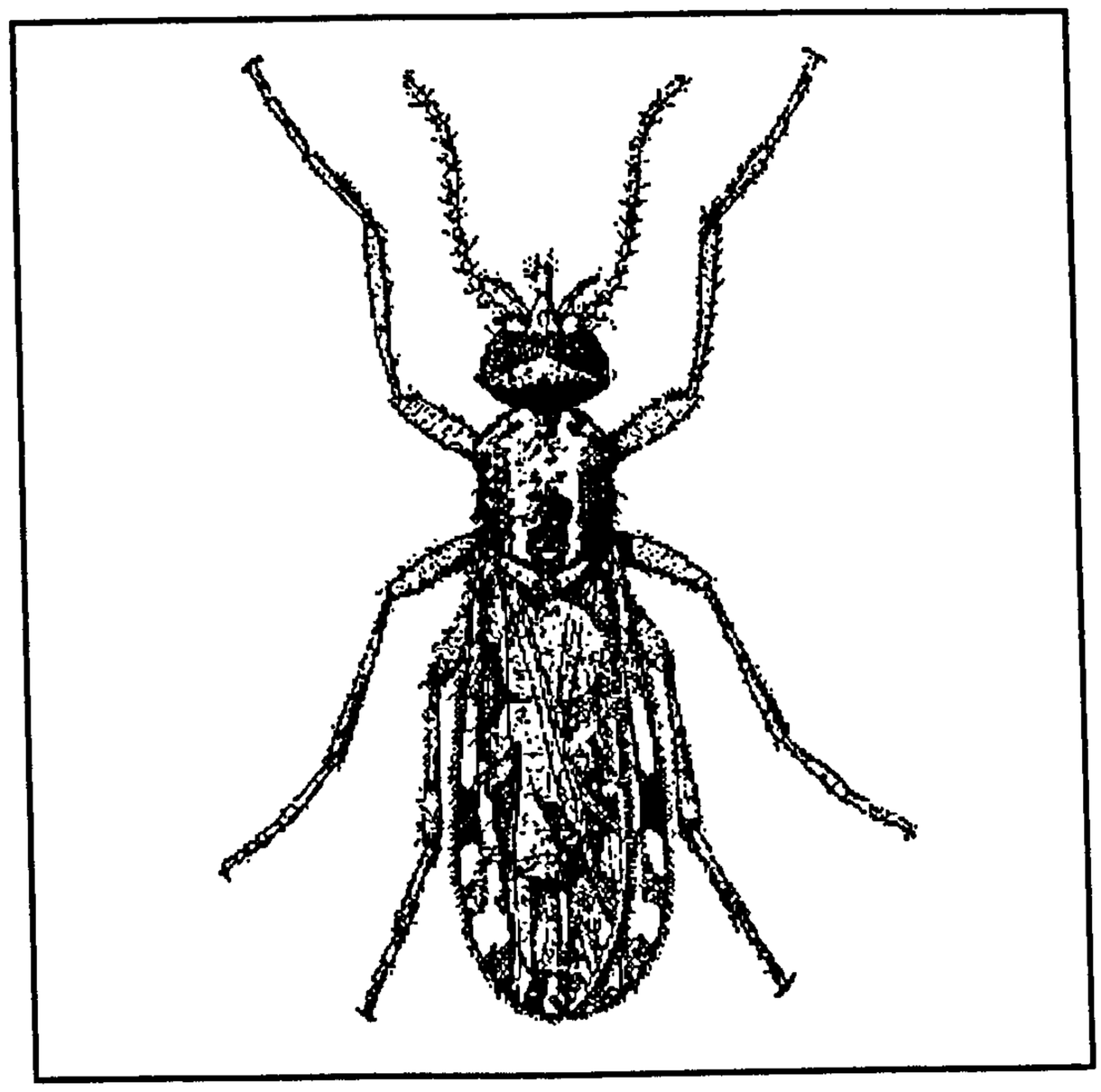

Figure 1.1 *Culicoides* spp. Adults are usually 1-3 mm in length, with grey and white patterned wings. Image obtained from Blanton and Wirth (1979).

and Phelps, 1981), although some species do have strong host preferences (Kettle, 1995).

In the majority of species adult activity is crepuscular and/or nocturnal (Kettle, 1995) and is greatest when evening and night-time conditions are warm, humid and calm (Boorman, 1993). *Culicoides* undertake flight to seek mates, blood-meals, shelter or suitable breeding sites. They usually only fly short distances from their larval habitat (Kettle, 1995), but can be carried on the wind for distances possibly up to 700 km (Sellers, 1992).

Economic importance

Culicoides species are of economic importance for two reasons. First, they can be a biting nuisance, limiting tourism and outdoor activities in many parts of the world (e.g. Scotland, USA, Caribbean, South America, Australia, and islands in the Pacific and

Indian Oceans; Kettle, 1995), while in horses *Culicoides* bites can cause a severe allergic dermatitis (sweet itch). Second, *Culicoides* have the capacity to transmit a range of pathogens and parasites to man and livestock. For example, *Culicoides* have been associated with the transmission of 12 species of protozoa and 18 species of filarial nematodes (Linley, 1985). However, it is as vectors of arboviruses (i.e. viruses transmitted to vertebrates by insects or acarines and which multiply in both vertebrate and invertebrate hosts) to livestock that *Culicoides* attain their full economic importance. Fifty-three viruses have been isolated from *Culicoides* (Meiswinkel *et al.*, 1994) and as mentioned previously two of these, BTV and AHSV, cause diseases of such international concern that they have OIE list 'A' status. This thesis focuses primarily on these two virus species.

1.3 Bluetongue Virus and African Horse Sickness Virus

BTV and AHSV are both double-stranded RNA viruses within the genus *Orbivirus* of the family Reoviridae. The virions, which are about 70 nm in diameter, consist of a core containing 10 segments of double-stranded RNA and composed of five proteins and an outer capsid made up of two further proteins (Mertens, 1994; Figure 1.2). At present 24 BTV serotypes and 9 AHSV serotypes are recognised.

Vertebrate Hosts

BTV infects all species of ruminants, although only causes severe disease in certain breeds of sheep (e.g. fine wool and mutton breeds; MacLachlan, 1994) and in some species of deer (Robinson *et al.*, 1967; Stair *et al.*, 1968). Mortality rates in sheep vary from 2-30% and the clinical symptoms include fever, depression, hyperaemia of buccal and nasal mucosae, nasal discharge, excess salivation, oedema of the head and neck, lameness, stiffness, coronitis and torticollis (Erasmus, 1990; Verwoerd and Erasmus, 1994; Geering *et al.*, 1995). Infection of pregnant ewes can also cause abortion or foetal abnormalities. Convalescent periods can be lengthy and sheep may shed their fleece (Geering *et al.*, 1995).

AHSV infects equids and occasionally dogs. Clinical signs result from the impaired function of the circulatory and respiratory systems and include serous effusions and haemorrhage in various organs and tissues (Howell, 1963). Mortality rates in horses

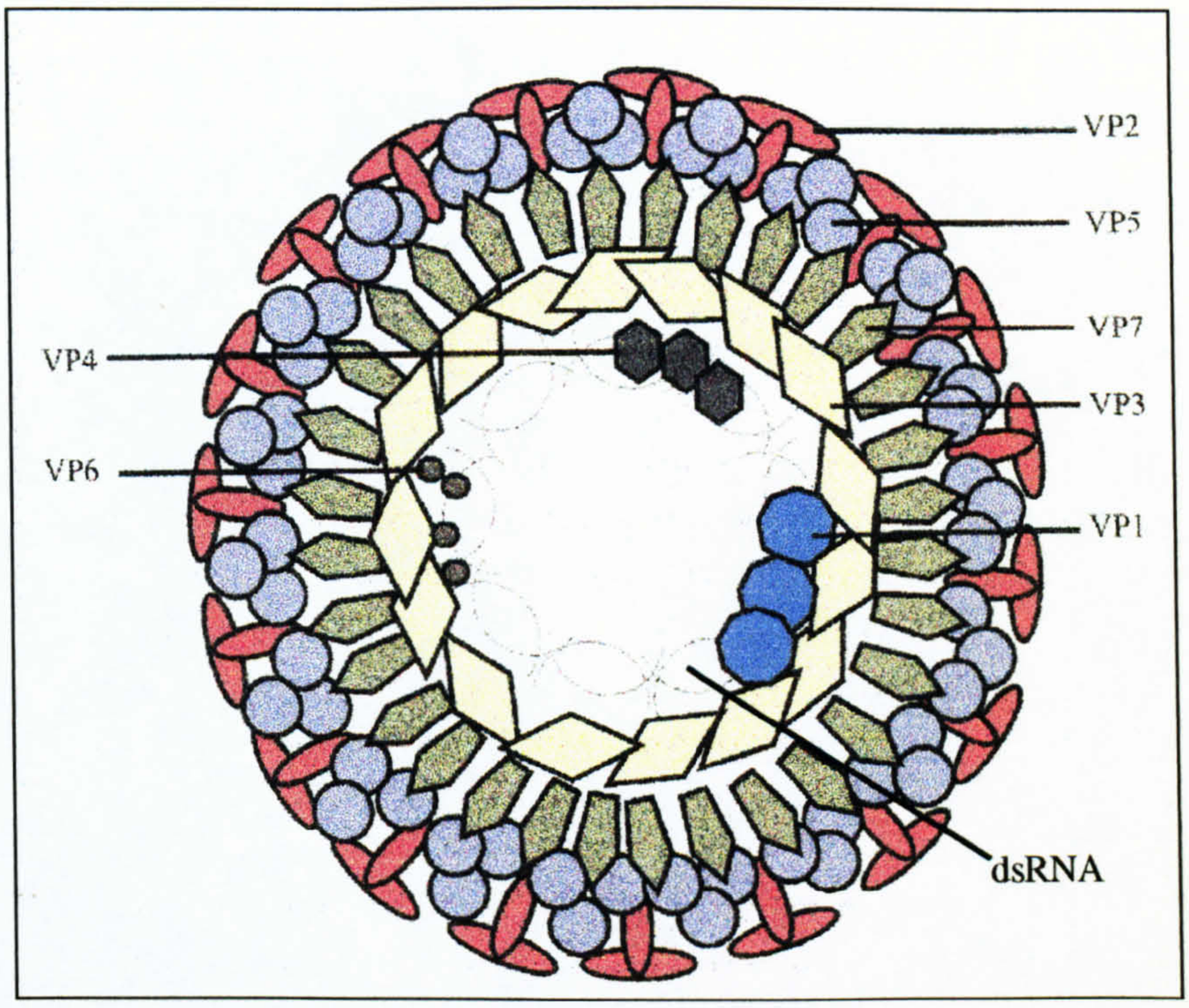

Figure 1.2 Schematic diagram of the organisation of the structural proteins of an orbivirus virion. The outer capsid is composed of structural proteins VP2 and VP5. The core consists of proteins VP7 and VP3, surrounding VP1, VP4 and VP6, as well as the double-stranded RNA. Image obtained from Thevasagayam (1998).

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vary from about 70% with serotype 9 to 95% with serotypes 1-8 (Coetzer and Erasmus, 1994), making it one of the most lethal horse diseases. However, the disease in donkeys and mules is less severe and in zebras clinical signs may be absent altogether (Mellor, 1994). Although the disease in dogs can be fatal, dogs are generally considered to be dead-end hosts, as *Culicoides* do not readily feed on them (e.g. Braverman and Chizov-Ginzburg, 1996) and they usually become infected by eating virus-contaminated horse meat.

Distribution

BTV occurs between latitudes of approximately 40°N to 35°S (Mellor, 1990; Mellor and Boorman, 1995), although in parts of North America it can occur up to almost 50°N (Dulac *et al.*, 1988; Dulac *et al.*, 1989). Within these areas, it is found in Africa,

Asia, North America, South America, Australia and some islands in the tropics and subtropics (Gibbs and Greiner, 1994). There have also been sporadic outbreaks in southern Europe, for example in Portugal and Spain (1956-60), (Campano Lopez and Sanchez Botija, 1958; Anon, 1960) and on the Greek islands of Lesbos (1979) (Vassalos, 1980) and Rhodes (1980) (Dragonas, 1981). The outbreak in Iberia was the most serious ever recorded and caused the deaths of more than 179,000 sheep (Campano Lopez and Sanchez Botija, 1958).

More recently (1998-1999) BTV was reported from the Greek Islands of Rhodes, Kos, Samos, Lesbos, Evia and Chios (Anon, 1998; Anon, 1999e; Anon, 2000b). Outbreaks of BTV also occurred for the first time in European Turkey (Anon, 1999c), mainland Greece (Anon, 1999d,e) and Bulgaria (Anon, 1999b). The outbreak in the Burgas region of Bulgaria (42-43°N) was in fact the furthest north that BTV has occurred in Europe.

AHSV is not as widely distributed as BTV and is usually confined to sub-Saharan Africa (Mellor, 1993). Its spread into North Africa and countries around the Mediterranean sea and Asia has been hindered by the Sahara Desert (Coetzer and Erasmus, 1994). However as with BTV, it is capable of making periodic excursions beyond its enzootic zone (Mellor, 1996). There have been two outbreaks of AHSV in Europe, which were in Spain in 1966 (Diaz Montilla and Panos Marti, 1968) and Spain and Portugal between 1987 and 1990 (Lubroth, 1988; Rodriguez *et al.*, 1992). In 1989, the virus also spread from Iberia into northern Morocco, where it persisted until 1991, so that in total the outbreak lasted five years. This persistence was unprecedented, as prior to this the longest that AHSV survived outside of its enzootic zone was 2-3 years (Mellor, 1998). The furthest north that AHSV has occurred is Madrid (40°N) (Lubroth, 1988).

Transmission

Culicoides were first implicated as vectors of BTV and AHSV by Du Toit (1944) (and see Wetzel *et al.*, 1970), when he demonstrated that *C. pallidipennis* (= *C. imicola*) was able to transmit BTV from infected to susceptible sheep, and AHSV from an infected to a susceptible horse. Although *Culicoides* are considered to be the principal

vector of these viruses, other arthropods have also been connected with BTV and AHSV. For example, the pajaroello tick *Ornithodoros coriaceus* has been shown to biologically transmit BTV to susceptible animals (Stott *et al.*, 1985), while the sheep ked *Melophagus ovinus* is a mechanical vector of BTV (Luedke *et al.*, 1965). *Anopheles stephensi*, *Culex pipiens* (Ozawa and Nakata, 1965), *Aedes aegypti* (Ozawa *et al.*, 1966), the camel tick *Hyalomma dromedarii* (Awad *et al.*, 1981), and the brown dog tick *Rhipicephalus sanguineus sanguineus* (Dardiri and Salama, 1988) have all been shown to biologically transmit AHSV to susceptible horses. In addition, trans-stadial transmission of AHSV from larvae to nymphs and from nymphs to adults has been demonstrated in the camel tick and the brown dog tick (Awad *et al.*, 1981; Dardiri and Salama, 1988). However, it is doubtful whether these additional species are involved in the maintenance and transmission of BTV and AHSV in the field.

To date there is no evidence for the transovarial transmission of BTV or AHSV by *Culicoides*. However, work in this area is limited (Jones and Foster, 1971; Nunamaker *et al.*, 1990). The inability of *Culicoides* to transovarially transmit the viruses is thought to be associated with the ovarial sheath (i.e. outer covering of the ovariole), which prevents the viruses from entering the developing eggs (Fu, 1996).

The lack of transovarial transmission of the viruses in *Culicoides*, combined with the relatively short duration of BT and AHS viraemias in infected vertebrate hosts (generally <30 days; Erasmus, 1990; Barnard *et al.*, 1994; Coetzer and Erasmus, 1994; Fassi-Fihri *et al.*, 1998; Hamblin *et al.*, 1998), means that adult *Culicoides* must be present for a large proportion of the year if the viruses are to persist in a region. Thus in enzootic areas, there must be continual cycles of transmission between the adult vectors and vertebrates, with any adult-free period being less than the maximum duration of the viraemia (Mellor, 1993, 1994). Winter conditions play a crucial role in determining whether the viruses can become enzootic, due to the impact of low temperatures on adult survivorship. For example, Sellers and Mellor (1993) found that *C. imicola* adults could only survive the winter (and the viruses therefore persist), in areas where the average daily maximum temperature during the coldest month of the year was ≥12.5°C.

Vector species

To date 24 *Culicoides* species have been associated with BTV, while 6 have been linked with AHSV. These figures include a) species from which BTV and AHSV have been isolated in the field b) species which have been shown to become infected with the viruses following the ingestion of a viraemic blood-meal and c) species which have been shown to biologically transmit the viruses (Tables 1.1 and 1.2). In addition, a further 5 species (*C. arakawae*, *C. circumscriptus*, *C. gemellus*, *C. shultzei* and *C. nudipalpis*) are suspected vectors of BTV, although as yet there is no evidence to confirm this (Dyce, 1989; Bi *et al.*, 1996; Meiswinkel and Baylis, 1998).

Culicoides imicola is considered to be the most important vector of BTV and AHSV in Europe (Mellor, 1990, 1996). This species is principally Afro-Asian, but in 1982 it was recorded for the first time in Europe, from Cordoba in southern Spain (Mellor *et al.*, 1983). It is now known to occur across most of south-western Iberia, up to 41°17'N in Portugal and 40°N in Spain (Rawlings *et al.*, 1997). In addition, it has been recorded from the Greek islands of Lesbos (Boorman and Wilkinson, 1983), Rhodes (Boorman, 1986), Chios, Kos, Samos (Mellor, pers. comm.) and Evia (Patakakkis, unpub. obs.). In 1999 it was also discovered on mainland Greece, in the provinces of Chaldithiki, Larisa and Magnisia (Patakakkis, unpub. obs.).

BTV has also been isolated from *C. obsoletus* in Cyprus (Mellor and Pitzolis, 1979) and AHSV has been recovered from mixed pools of *C. obsoletus* and *C. pulicaris* in Spain (Mellor *et al.*, 1990). Both species are widely distributed in Europe (Mellor, 1987). However, their role in disease transmission is unclear. For example, in Spain they are considered to be unimportant since they are only present in low numbers during the months when BTV and AHSV outbreaks typically occur (Ortega *et al.*, 1998). In contrast, during the 1999 BTV outbreak in Bulgaria, *C. obsoletus* was the most numerous species trapped at infected sites, while *C. imicola* was absent (Mellor, pers. comm.).

Also present in Europe are *C. impunctatus* and *C. nubeculosus*. These species have been orally infected with BTV in the laboratory, although the infection rates (i.e. proportion of individuals susceptible to the virus) were very low (Jennings and Mellor,

Table 1.1 *Culicoides* species associated with BTV.

Subgenus	Species	Virus Isolation	Oral Infection	Transmission	Reference
Avaritia	*C. actoni*		+	+	Standfast *et al.* (1985)
	C. bolitinos	+	+		Barnard *et al.* (1998) Venter *et al.* (1998)
	C. brevipalpis		+		Standfast *et al.* (1985)
	C. brevitarsis	+	+	+	Muller *et al.* (1982) St George & Muller (1984) Muller (1985) Standfast *et al.* (1985) Muller (1987) Bellis *et al.* (1994)
	C. fulvus	+	+	+	Standfast *et al.* (1979) Standfast *et al.* (1985)
	C. gulbenkiani (= *C. tororoensis*)	+			Walker & Davies (1971)
	C. imicola	+	+	+	Du Toit (1944) Nevill *et al.* (1992) Venter *et al.* (1991) Venter *et al.* (1998)
	C. obsoletus	+	+		Mellor & Pitzolis (1979) Mellor & Jennings (1986)
	C. orientalis	+			Sendow *et al.* (1993)
	C. pusillus	+			Mo *et al.* (1994)
	C. wadai	+	+		Standfast *et al.* (1985) Bellis *et al.* (1994) McColl *et al.* (1994)
Beltranmyia	*C. pycnostictus*	+			Nevill *et al.* (1992)
Culicoides	*C. magnus*		+		Venter (pers. comm.)
	C. impunctatus		+		Mellor & Jennings (1986) Jennings & Mellor (1988)
Diphaeomia	*C. debilipalpis*		+		Mullen *et al.* (1985)
	C. stellifer	+			Mullen & Anderson (1998)
Hoffmania	*C. insignis*	+	+	+	Greiner *et al.* (1985) Tanya *et al.* (1992)
	C. milnei	+			Walker & Davies (1971)

Table 1.1 Continued.

Subgenus	Species	Virus Isolation	Oral Infection	Transmission	Reference
Hoffmania	*C. peregrinus*		+		Standfast *et al.* (1985)
	C. venustus		+		Jones *et al.* (1983)
Monoculicoides	*C. nubeculosus*		+	+	Mellor & Boorman (1980) Mellor & Jennings (1986) Jennings & Mellor (1988) Chapter 5
	C. variipennis	+	+	+	Foster *et al.* (1968) Foster & Jones (1973) Jones & Foster (1978) Jones *et al.* (1981)
Oecacta	*C. oxystoma*		+		Standfast *et al.* (1985)
Similis group	*C. exspectator*	+			Nevill *et al.* (1992)

Table 1.2 *Culicoides* species associated with AHSV.

Subgenus	Species	Virus Isolation*	Oral Infection	Transmission	Reference
Avaritia	*C. bolitinos*	+	+		Meiswinkel & Paweska (1998) Venter *et al.* (in press)
	C. imicola	+	+	+	Du Toit (1944; cited in Wetzel *et al.*, 1970) Nevill *et al.* (1992) Venter *et al.* (in prep.)
	C. obsoletus	+			Mellor *et al.* (1990)
Culicoides	*C. pulicaris*	+			Mellor *et al.* (1990)
Monoculicoides	*C. nubeculosus*	+			Mellor *et al.* (1998) Chapter 5
	C. variipennis	+	+		Boorman *et al.* (1975) Mellor *et al.* (1975) Wellby *et al.* (1996)

*The isolation of AHSV from *C. gulbenkiani* reported by Meiswinkel *et al.* (1994) was incorrect (Meiswinkel, 1997).

1988). However, the simultaneous infection of *C. nubeculosus* with BTV and *Onchocerca cervicalis* microfilariae can enhance its susceptibility (Mellor and Boorman, 1980). Furthermore, the susceptibility of this species to AHSV is greatly increased when the immature stages are initially exposed to elevated temperatures (Mellor *et al.*, 1998).

1.4 *CULICOIDES* AS A VECTOR

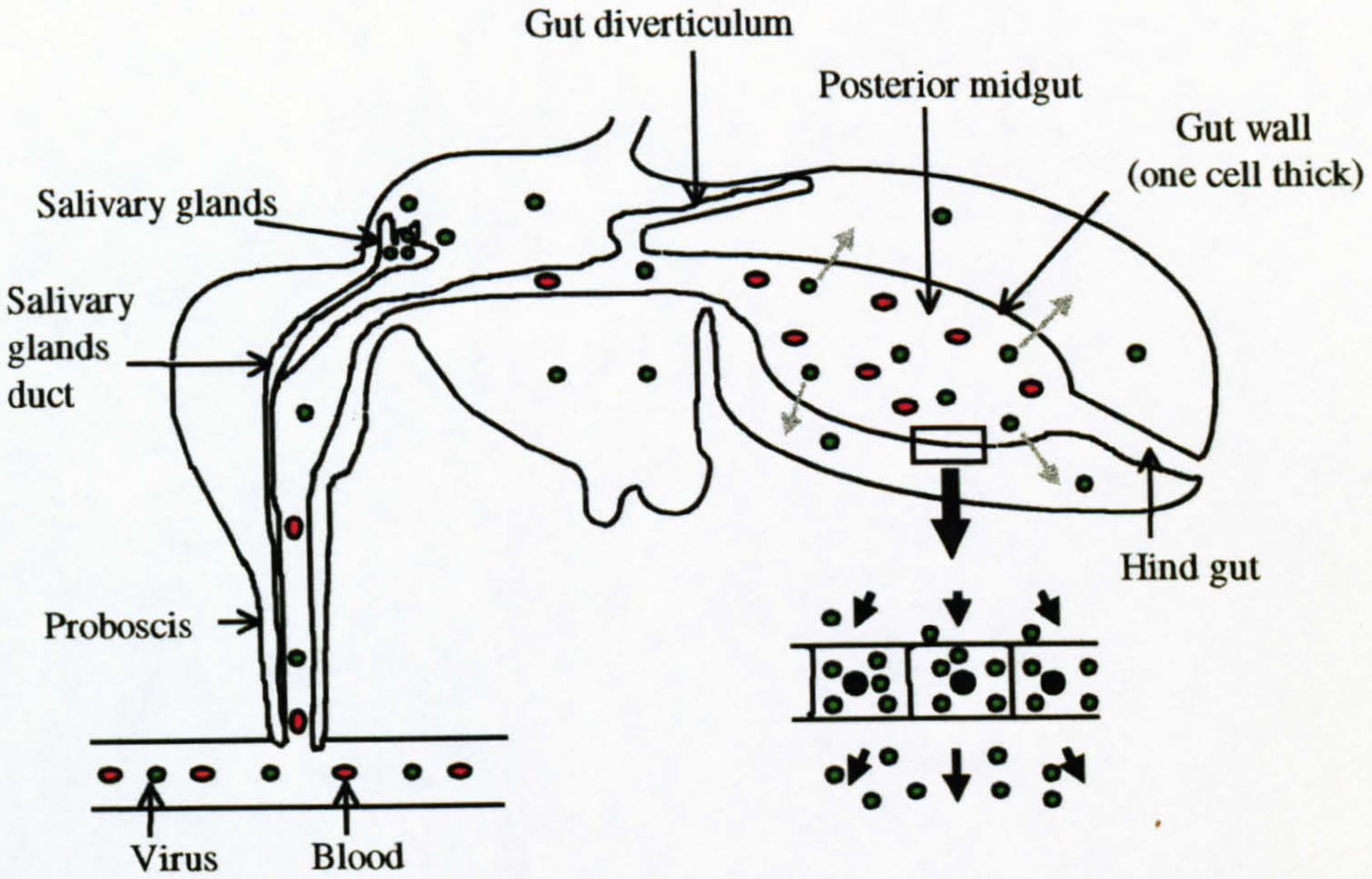
Oral infection

In the wild, vector *Culicoides* become infected with arboviruses when they ingest a blood-meal from a viraemic vertebrate host. The blood and virus are deposited into the posterior region of the insect's midgut. The virus particles then attach themselves to the luminal surface of the gut cells, infect these cells and then replicate in them. Progeny virions are released through the basement membrane to the haemocoel where they infect secondary target organs, including the salivary glands. After replication in the salivary glands, viral transmission can occur during subsequent biting activity (Mellor, 1990; Figure 1.3a). The bite from a single midge is sufficient to infect a susceptible vertebrate host (e.g. Foster *et al.*, 1968).

Individuals that have the ability to become infected with a virus after ingestion of a viraemic blood-meal and to subsequently transmit virus by bite are classed as 'vector competent'. The interval between virus ingestion and the subsequent ability to transmit virus is termed the extrinsic incubation period (EIP). The duration of the EIP is dependent on temperature and takes about 10 days at 25°C (Mullens *et al.*, 1995; Wellby *et al.*, 1996).

However, even within a vector species of *Culicoides*, only a proportion of individuals are likely to be competent to transmit a particular arbovirus (Jones and Foster, 1978; Jennings and Mellor, 1987). Individuals that are refractory to infection may possess a midgut infection barrier, where virus is unable to enter the midgut cells, or a midgut escape barrier, where virus can replicate in the midgut cells but is unable to exit into the haemocoel (Jennings and Mellor, 1987). Fu *et al.* (1999) have also reported the existence of a dissemination barrier, where virus that enters the haemocoel is unable to infect secondary target organs (Figure 1.3b).

a.



b.

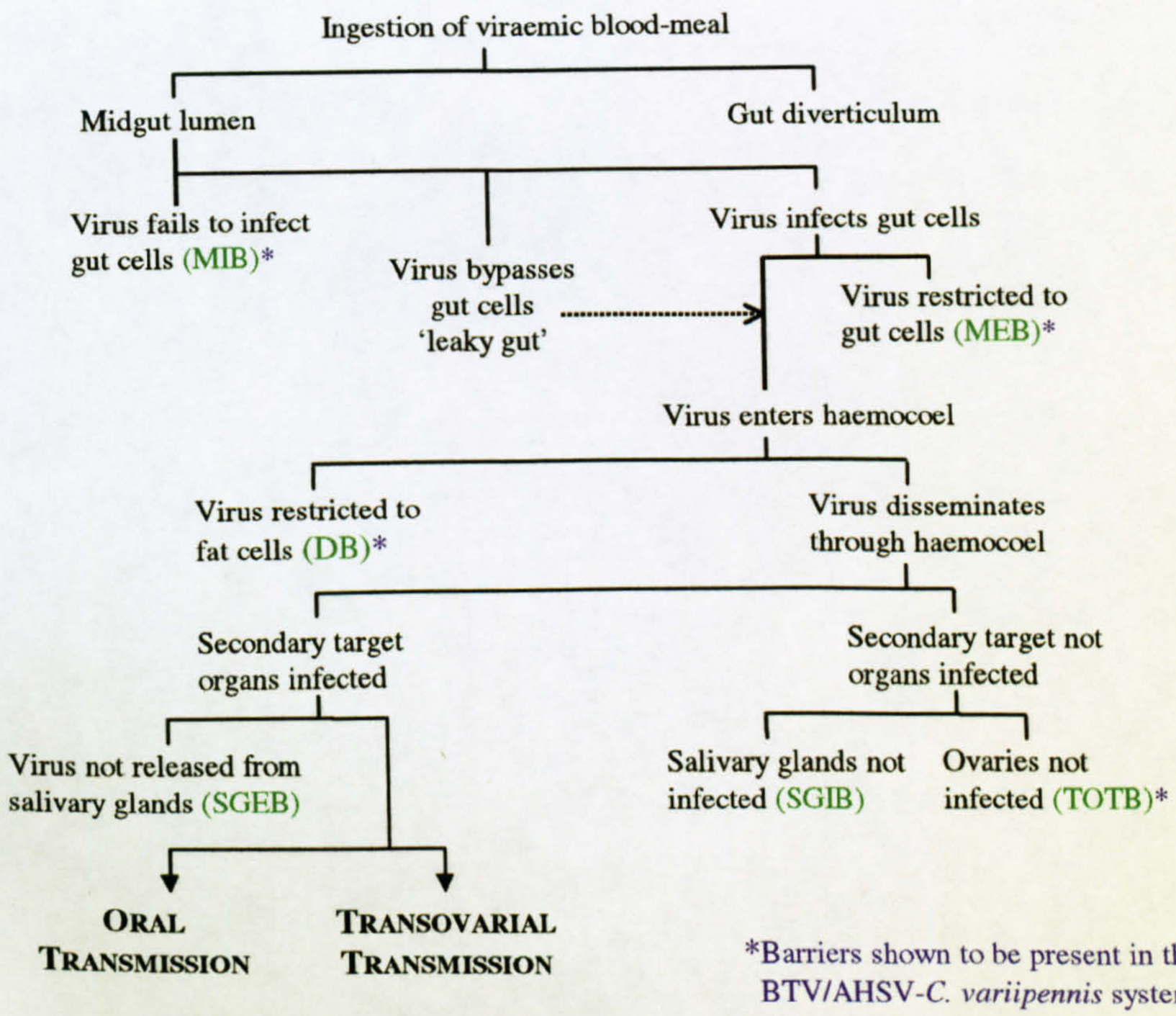


Figure 1.3 Arbovirus infection of haematophagous insects. (a) Schematic of the cycle of an arbovirus in a vector. (b) Hypothesised barriers to arbovirus infection in haematophagous insects. MIB = midgut infection barrier, MEB = midgut escape barrier, DB = dissemination barrier, TOTB = transovarial transmission barrier, SGIB = salivary gland infection barrier and SGEB = salivary gland escape barrier. (Adapted from Mellor *et al.*, 2000).

These barriers are under genetic control and Tabachnick (1991) demonstrated that the susceptibility of *C. variipennis* to BTV was determined by a single gene, with a major locus and a modifier. The major locus acts via a maternal effect (i.e. the maternal genotype determines the progeny phenotype) and paternal imprinting (i.e. the paternal gene is dominant in the offspring). In addition, the susceptibility of *Culicoides* to arboviruses may be influenced by extrinsic factors, including temperature (Mullens *et al.*, 1995; Wellby *et al.*, 1996; Mellor *et al.*, 1998).

Vectorial capacity

The ability of a *Culicoides* population to transmit a virus to a vertebrate population can be assessed by determining its vectorial capacity (*C*), according to the following equation:

$C = ma^2Vp^n / (-\ln p)$

where C = number of new infections per case per day, m = the number of vectors per host, a = number of blood-meals taken by a vector per host per day, V = vector competence, p = daily survival rate of the vector, and n = extrinsic incubation period in days (Mullens, 1992). All of these parameters can be affected by the ambient conditions.

1.5 Climate and *Culicoides*

The spatial and temporal incidence of BTV and AHSV are affected by the distribution and vectorial capacity of *Culicoides* vector populations. In turn, these are influenced by climatic factors such as temperature, precipitation, humidity and wind. Of these factors, temperature has the greatest influence on virus transmission and our current knowledge in this area is described below. However, there are several aspects of the relationship between temperature and virus transmission which are poorly understood. If we wish to gain insight into the impact of climate change on the viral diseases, it is essential that these aspects are investigated further.

Temperature and distribution of Culicoides

The geographic ranges of most insect species are influenced by temperature and low temperatures tend to be more significant than high temperatures as determinants of

distribution (Gates, 1993). For example, there is some evidence that the northern limit of *C. imicola* in Iberia is determined by low temperature (Baylis and Rawlings, 1998; Rawlings *et al.*, 1998), although the exact temperature requirements of this species have not yet been established. However, there is concern there are areas of Europe that, while being climatically suitable, have yet to be colonised by *C. imicola* (Rawlings *et al.*, 1998). Understanding the relationship between temperature and the distribution of *C. imicola* is therefore critical if we are to determine where this vector could become established in Europe. Additionally, these data could be used to determine where *C. imicola* could occur if conditions warm with climate change (e.g. 2°C increase in the mean annual temperature corresponds to a northward shift of ≈200 km; Hughes, 2000).

Temperature and vectorial capacity

Temperature can influence the vectorial capacity of a *Culicoides* population both through changes in the overall size of the adult population and in the proportion of adults within the population capable of transmitting virus.

Adult population size

Very few midges ever transmit virus due to the low probability that they will a) initially feed on a viraemic host, b) be competent to transmit virus, c) survive the viral EIP and d) subsequently feed on a susceptible host. Nevertheless, while the chance of a given individual satisfying all these criteria is low (e.g. only 1 in 35000 female *C. brevitarsis* could transmit BTV; Muller *et al.*, 1982), this can be compensated for by the potentially huge midge population sizes (e.g. up to one million *C. imicola* may be caught in a single light trap in a single night; Meiswinkel, 1998). Temperature can greatly affect the size of an adult population (and hence the potential for viral transmission) through its impact on a) recruitment of adults from the immature stages b) adult survivorship and c) adult activity.

The influence of temperature on recruitment of adults is two-fold. First, the development rate of *Culicoides* from the egg to the adult stage has been shown to be directly related to temperature (within favourable limits; e.g. Edwards, 1982; Kitaoka, 1982; Mullens and Rutz, 1983; Vaughan and Turner, 1987; Bishop *et al.*, 1996). Thus

the warmer the weather, the shorter the life cycle and the greater the number of generations and adults that can be produced in a season. Second, survivorship of the immatures to adulthood is influenced by temperature and there is usually an optimal range of temperatures where survivorship is maximised (Kitaoka, 1982; Bishop *et al.*, 1996). Recruitment of adults is therefore greatest at temperatures where development is fast and immature survivorship is high and is limited at temperatures where either one or both of these factors are reduced.

However, no work has been carried out into the influence of temperature on the development of European *Culicoides* vector species. Both the optimum and lowest temperatures for immature development should be established. The former will give an indication of the times of year when recruitment of adults will be greatest and hence periods of risk for virus transmission. The latter will give an indication of whether development and therefore adult vectors could occur throughout the year, which is critical for the persistence of the viruses. Ideally, such work would involve *C. imicola*, as well as a Palaearctic *Culicoides* species. However, *C. imicola* has yet to be successfully colonised and a suitable model species must therefore be used in its place.

Survival of adult *Culicoides* is adversely affected by high temperatures (Hunt *et al.*, 1989; Wellby *et al.*, 1996) and this is considered further below. The frequency of key adult activities such as mating, host-seeking, blood-feeding and oviposition can affect the population growth rate. Warm conditions generally increase activity (Walker, 1977; Blackwell, 1997; Kettle *et al.*, 1998), while temperatures below 10°C for *C. variipennis* (Nelson and Bellamy, 1971) and 18°C for *C. brevitarsis* (Murray, 1987) inhibit activity.

Proportion of vectors

Temperature can influence the proportion of adult *Culicoides* capable of transmitting virus by its impact on the biting rate, adult survival, duration of the EIP and vector competence.

The biting rate is a critical factor in vectorial capacity since females must take a minimum of two blood meals to transmit virus - the first to acquire the virus and the second, after the completion of the EIP, to transmit the virus. Since female *Culicoides*

generally require a blood meal for every batch of eggs they mature, the biting rate is largely governed by the time required for the eggs to develop (gonotrophic cycle). High temperatures have been shown to reduce the duration of the gonotrophic cycle (Linley, 1966; Mullens and Holbrook, 1991) and thereby increase the biting rate. For example, female *C. variipennis sonorensis* blood-feed every 3 days at 30°C and only every 14 days at 13°C (Mullens and Holbrook, 1991).

The relationship between adult survival and the duration of the EIP is also important in determining vectorial capacity, since transmission cannot occur unless females live long enough to blood-feed after the completion of the viral EIP. However these factors are conversely affected by temperature. For example, at high temperatures vector survivorship is reduced but virus development is rapid, whereas at low temperatures survivorship is extended but virogenesis is slow (Hunt *et al.*, 1989; Mullens *et al.*, 1995; Wellby *et al.*, 1996). It is therefore necessary to establish at which temperatures females are most likely to survive long enough to complete the EIP, as well as the range of temperatures over which this can occur.

Temperature can also influence the vector competence of *Culicoides* vectors. For example, BTV11 and AHSV9 are unable to develop in *C. variipennis sonorensis* at temperatures below about 15°C (Mullens *et al.*, 1995; Wellby *et al.*, 1996). Hence even if a midge is genetically capable of transmitting the virus, it will be unable to do so at temperatures below this limit. In addition, within the range of temperatures where virus replication may occur, Wellby *et al.* (1996) found that the infection rate of *C. variipennis sonorensis* with AHSV9 increased with temperature. However, the ability of a midge to become infected with a virus (i.e. virus can replicate in the midgut) does not necessarily mean that it will be competent to transmit the virus, as some midges possess a midgut escape barrier (where virus is restricted to the midgut cells; Jennings and Mellor, 1987) or a dissemination barrier (where virus is unable to infect secondary target organs; Fu *et al.*, 1999). It is therefore necessary to establish whether vector competence could also increase with temperature. However, Mullens *et al.* (1995) found that the infection rate of *C. variipennis sonorensis* with BTV11 was similar at temperatures between 21-32°C. Hence it must also be determined whether the impact of temperature on vector competence varies with the virus species and serotype.

Temperature can even affect the competence of 'non-vector' *Culicoides* species. *Culicoides nubeculosus* is generally considered to be incapable of transmitting BTV and AHSV due to a midgut infection barrier (Mellor *et al.*, 1975; Mellor and Boorman, 1980). However, in a preliminary investigation Mellor *et al.* (1998) showed that a 5-10°C rise in the immature rearing temperature, from the standard rearing temperature of 25°C, could increase the oral infection rate of *C. nubeculosus* for AHSV from <1% to >10%. They suggested that this phenotypic change could result from a 'leaky midgut', where virus can leak directly into the haemocoel, bypassing the midgut barriers. Once in the haemocoel the virus can replicate and be transmitted even by what is normally considered to be a non-vector species. However, these results must be confirmed using larger sample sizes, while it is also necessary to establish if vector competence for BTV in *C. nubeculosus* can be induced by elevated rearing temperatures. In addition, it is important to determine the lowest rearing temperature at which vector competence can be induced and whether hot conditions for only part of the life cycle can have the same effect.

The impact of temperature on virus transmission by *Culicoides* is summarised in Figure 1.4.

Other climatic factors

Precipitation can influence the distribution and abundance of *Culicoides* species, through its affect on the availability of breeding sites. For example, *C. imicola* breeds in wet, organically enriched, soil or mud (Walker and Davies, 1971; Braverman *et al.*, 1974; Lubega and Khamala, 1976; Walker, 1977; Braverman, 1978), and in Africa it tends to occur in areas with rainfall of 300-700 mm per year (Meiswinkel and Baylis, 1998). Areas with >700 mm rain/annum are probably unsuitable as *C. imicola* pupae drown when breeding sites are flooded (Nevill, 1967). Furthermore, within a site in South Africa, Nevill (1971) found that the seasonal abundance of adult *C. imicola* was related to the amount of rainfall in the preceding month and that the greatest annual abundance occurred in the year with the greatest rainfall. Across sites in Morocco and Iberia, the annually-averaged mean daily catch of *C. imicola* has been related to the annual minimum Normalised Difference Vegetation Index (a measure of photosynthetic

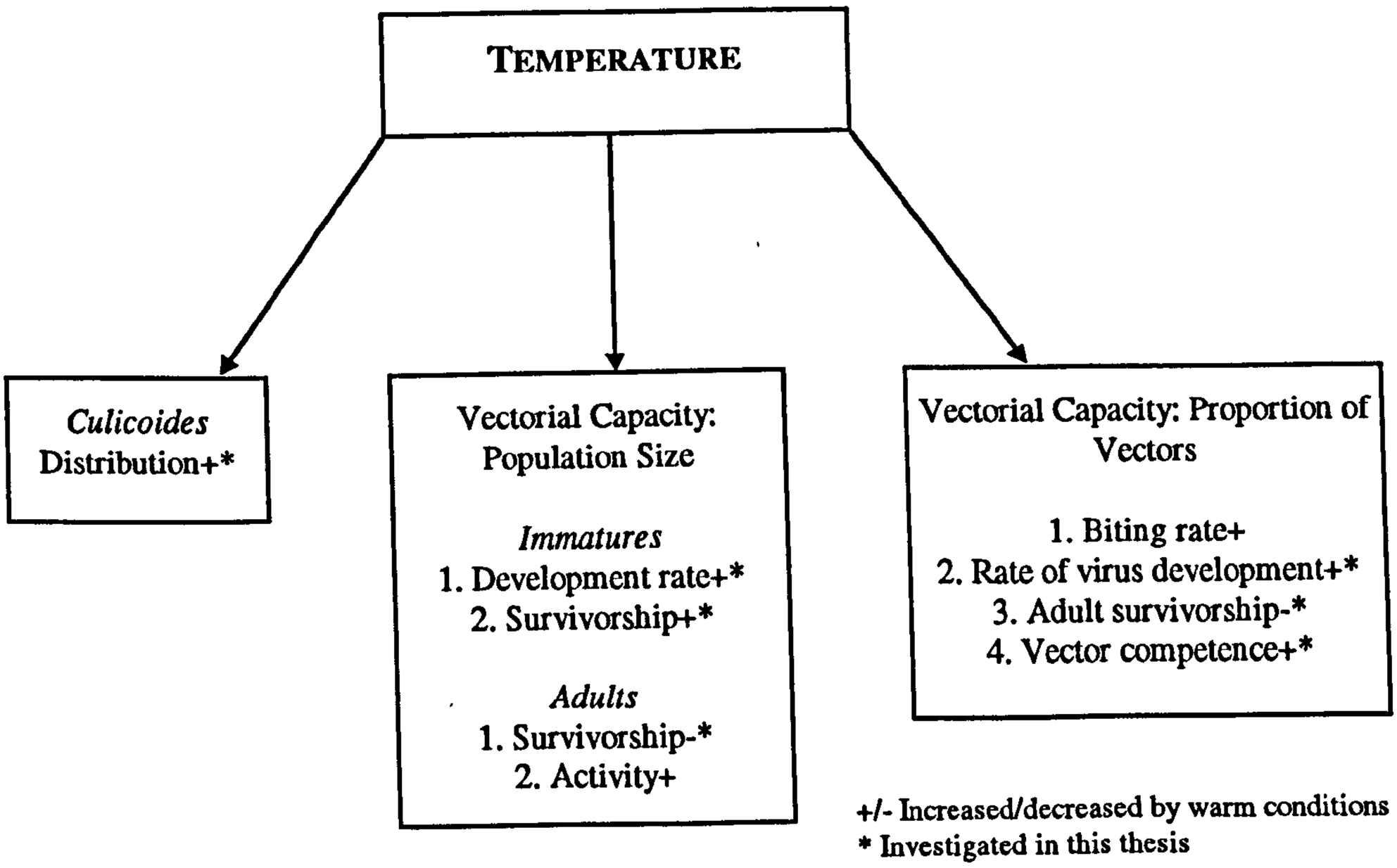


Figure 1.4 Influence of temperature on the transmission of BTV and AHSV by *Culicoides*.

activity which strongly correlates with soil moisture; Baylis and Rawlings, 1998; Baylis *et al.*, 1998a).

Humidity can affect the survival of adult *Culicoides*. Due to their small size adult midges are particularly susceptible to desiccation and Murray (1991) found that even brief periods at low humidities reduced the longevity of *C. brevitarsis*. Humidity can also positively affect the level of adult activity (Murray, 1975; Walker, 1977; Blackwell, 1997).

Wind speed and direction can affect *Culicoides* distribution, through their influence on the passive dispersal of the adults. Due to their small size, *Culicoides* are exceptionally susceptible to this means of dispersal. In winds at speeds of 10-40 km/h, at heights up to 1.5 km and at temperatures between 12 and 35°C, *Culicoides* may be carried as aerial plankton for distances up to 700 km (Sellers, 1992). This long distance dispersal

can result in *Culicoides* species colonising new areas (Dyce, 1982) and in the introduction of virus-infected *Culicoides* into previously disease free regions. Significantly, the majority of BTV and AHSV outbreaks in Europe have been attributed to the wind-carriage of infected *Culicoides* (Sellers *et al.*, 1977; Sellers *et al.*, 1978; Boorman and Wilkinson, 1983; Mellor, 1987; Anon, 1999a,b).

There is also some evidence that wind can adversely affect adult survival. Baylis *et al.* (1998a, b) found that the mortality rate of *C. imicola* at eight sites in Morocco was positively correlated with wind speed, but was not affected by temperature, relative humidity or saturation deficit. However, it is not known whether the midges were actually killed at the windier sites or simply dispersed. Wind also negatively affects activity (Walker, 1977; Blackwell, 1997; Kettle *et al.*, 1998), which is suppressed at wind speeds greater than 3 m/s for *C. imicola* in Kenya (Walker, 1977) and 2.2 m/s for *C. brevitarsis* in Australia (Murray, 1987).

In summary, climatic factors such as temperature, precipitation, humidity and wind are critical in determining the distribution and vectorial capacity of *Culicoides* populations. These in turn will affect the spatial and temporal incidence of BTV and AHSV. However, there are several aspects of the relationship between climate and virus transmission which are poorly understood. If we wish to gain insight into the impact of climate change on BTV and AHSV, it is essential that these aspects are investigated further.

1.6 Thesis Plan

In this thesis I begin by describing how temperature affects the development of the immature stages of the northern European midge, *C. nubeculosus* and the North American midge, *C. variipennis sonorensis* (Chapter 2). The latter species was used as a model for *C. imicola*. I then report on how temperature influences the transmission of orbiviruses (BTV, AHSV and epizootic haemorrhagic disease virus) by *C. variipennis sonorensis*, through its impact on the EIP, vector competence and adult survival (Chapter 3). I also present the results of the first study into the influence of temperature on the transmission of AHSV by a field population of *C. imicola* (Chapter 4). While Chapters 2-4 focus primarily on the influence of temperature on *Culicoides*

vector species, in Chapter 5 I describe a special case, whereby non-vector species can become vectors when exposed to high temperatures during their development. I investigate this phenomenon using *C. nubeculosus* infected with BTV and AHSV. In chapter 6 I identify areas of Europe that have suitable climates for the occurrence of *C. imicola*, both currently and if conditions warm with climate change. Finally, I summarise the results and consider how climate change will alter the risk of BTV and AHSV in Europe (Chapter 7).

CHAPTER 2: EFFECT OF TEMPERATURE ON THE DEVELOPMENT OF THE IMMATURE STAGES OF *CULICOIDES* BITING MIDGES (DIPTERA: CERATOPOGONIDAE) AND IMPLICATIONS FOR THE TRANSMISSION OF BLUETONGUE VIRUS AND AFRICAN HORSE SICKNESS VIRUS.

2.1 ABSTRACT

The size of an adult *Culicoides* population is dependent on recruitment from the immature stages and adult survivorship. Here, I investigated the effect of temperature on the development of immature *C. nubeculosus* and *C. variipennis sonorensis*. The development rate of both species increased linearly between 12.5 and 35°C, while immature survivorship was greatest at rearing temperatures of 25 and 30°C for *C. nubeculosus*, and at 25°C for *C. variipennis sonorensis*. The optimum temperature range for development, based on both development rate and immature survivorship, was 25-30°C for *C. nubeculosus* and 25-35°C for *C. variipennis sonorensis*, while the minimum temperatures for development were estimated to be 8.1°C and 10.7°C, respectively. Temperatures experienced during the immature stages did not affect survival of adult *C. variipennis sonorensis*, when adults were maintained at a range of temperatures. As *Culicoides* species are the major vectors of bluetongue virus and African horse sickness virus and virus transmission is greatest when there are large numbers of vectors, such data will prove useful in predicting where disease outbreaks are most likely to occur, both currently and if conditions should warm with climate change.

2.2 INTRODUCTION

Bluetongue virus (BTV) and African horse sickness virus (AHSV) are both dsRNA viruses within the genus *Orbivirus* of the family Reoviridae. BTV infects all species of ruminants, causing severe disease (bluetongue; BT) in certain breeds of sheep (MacLachlan, 1994) and in some species of deer (Robinson *et al.*, 1967; Stair *et al.*, 1968). AHSV infects equids and the disease (African horse sickness; AHS) is most devastating in horses, with mortality ranging from 70 to 95% (Coetzer and Erasmus, 1994). Indeed, BT and AHS are of such major international concern that they have been designated as OIE list 'A' diseases.

BTV and AHSV are transmitted between their respective vertebrate hosts by biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae). In general, outbreaks of BT and AHS tend to occur at times of year when numbers of adult *Culicoides* vectors reach their peak (Mellor and Boorman, 1995; Mellor, 1996). Modelling studies have also indicated that the size of an adult *Culicoides* vector population is critical in determining both the likelihood and severity of an outbreak, following the introduction of virus (Lord *et al.*, 1996).

The presence of adult *Culicoides* vectors (even in low numbers) is also critical in determining the persistence of BTV or AHSV in a region. Due to the lack of transovarian transmission of the viruses in *Culicoides* (Jones and Foster, 1971; Nunamaker *et al.*, 1990) and the relatively short duration of BT and AHS viraemias in infected vertebrate hosts (generally <30 days; Erasmus, 1990; Barnard *et al*, 1994; Coetzer and Erasmus, 1994; Fassi-Fihri *et al.*, 1998; Hamblin *et al.*, 1998), adult *Culicoides* vectors must be present for a large proportion of the year if the viruses are to persist. Thus there must be continual cycles of transmission between the adult vectors and vertebrates, with any adult-free period being less than the maximum duration of the viraemia (Mellor, 1993, 1994).

The abundance and seasonality of adult *Culicoides* vectors are largely dependent on recruitment from the developing immatures, which in turn is influenced by temperature. For example, the development rates of Australian populations of *C. brevitarsis* (Allingham, 1991; Bishop *et al.*, 1996) and *C. subimmaculatus* (Edwards, 1982), Japanese populations of *C. arakawae* and *C. maculatus* (Kitaoka, 1982), and of a North American species complex, *C. variipennis* (Mullens and Rutz, 1983; Vaughan and Turner, 1987), were found to be faster at higher temperatures. Thus the warmer the weather, the shorter the life cycle and the greater the number of generations that could be produced in a season. In contrast, however, high temperatures can reduce survivorship of the immatures to adulthood (Kitaoka, 1982; Bishop *et al.*, 1996). Thus recruitment of adults is greatest at temperatures warm enough for rapid development but cool enough for high immature survivorship (optimum temperature range). At temperatures which are either colder or hotter than the optimum, recruitment of adults is reduced.

A number of outbreaks of BT and AHS have occurred in southern Europe (Campano Lopez and Sanchez Botija, 1958; Diaz Montilla and Panos Marti, 1968; Vassalos, 1980; Dragonas, 1981; Rodriguez *et al.*, 1992; Anon, 1998; Anon, 1999b,c,d,e; Anon, 2000b). Consequently, in order to identify times of midge emergence and periods of risk for virus transmission in Europe, both currently and if conditions should warm (as predicted by climate change scenarios; Intergovernmental Panel on Climate Change, 1996), it would be advantageous to quantify the effect of temperature on the development of European *Culicoides* vector species.

In this chapter, I investigate the effect of temperature on the development of the northern European midge, *C. nubeculosus*. However, this species is generally considered to be incapable of transmitting BTV or AHSV (Mellor *et al.*, 1975; Mellor and Boorman, 1980), except under unusual circumstances (Mellor and Boorman, 1980; Mellor *et al.*, 1998; Chapter 5). Nevertheless, given its distribution, *C. nubeculosus* can be used as a model for other Palearctic species, such as *C. obsoletus* and *C. pulicaris*, which have been implicated in the transmission of these viruses (Mellor and Pitzolis, 1979; Mellor *et al.*, 1990). However, the main advantage of working with *C. nubeculosus* is that it has been colonised in the laboratory (Boorman, 1974).

The most important vector species of BTV and AHSV in Europe is *C. imicola* (Mellor, 1990; Mellor, 1996). This species is principally Afro-Asian, but has also been found in south-western Iberia (Rawlings *et al.*, 1997), mainland Greece (Patakkakis, unpub. obs.) and the Greek islands of Lesbos (Boorman and Wilkinson, 1983), Rhodes (Boorman, 1986), Chios, Kos, Samos (Mellor, pers. comm.) and Evia (Patakkakis, unpub. obs.). However, *C. imicola* has proved impossible to rear in the laboratory. Hence to provide some insight into the effect of temperature on the development of *Culicoides* species, that originate from warmer climates than those experienced in Europe, a laboratory colony of *C. variipennis sonorensis* (Boorman, 1974), the major vector of BTV in North America (Mellor, 1990), was used. Two previous studies have reported the influence of temperature on the development of *C. variipennis* (Mullens and Rutz, 1983; Vaughan and Turner, 1987). However, there are methodological problems associated with both and I consider this further in the discussion.

The present study was therefore carried out to determine the effect of temperature on the development rate and immature survivorship of *C. nubeculosus* and *C. variipennis sonorensis*, in order to establish both the optimum rearing temperature and the range of temperatures over which successful development can occur. In addition, since survival of adult *Culicoides* also affects adult population size, the effect of the immature rearing temperature on adult survival was investigated.

2.3 METHODS

Development rate and immature survivorship trials were carried out at 12.5, 15, 20, 25, 30 and 35°C with a 24:0 (L:D) photoperiod, for both *C. nubeculosus* and *C. variipennis sonorensis*.

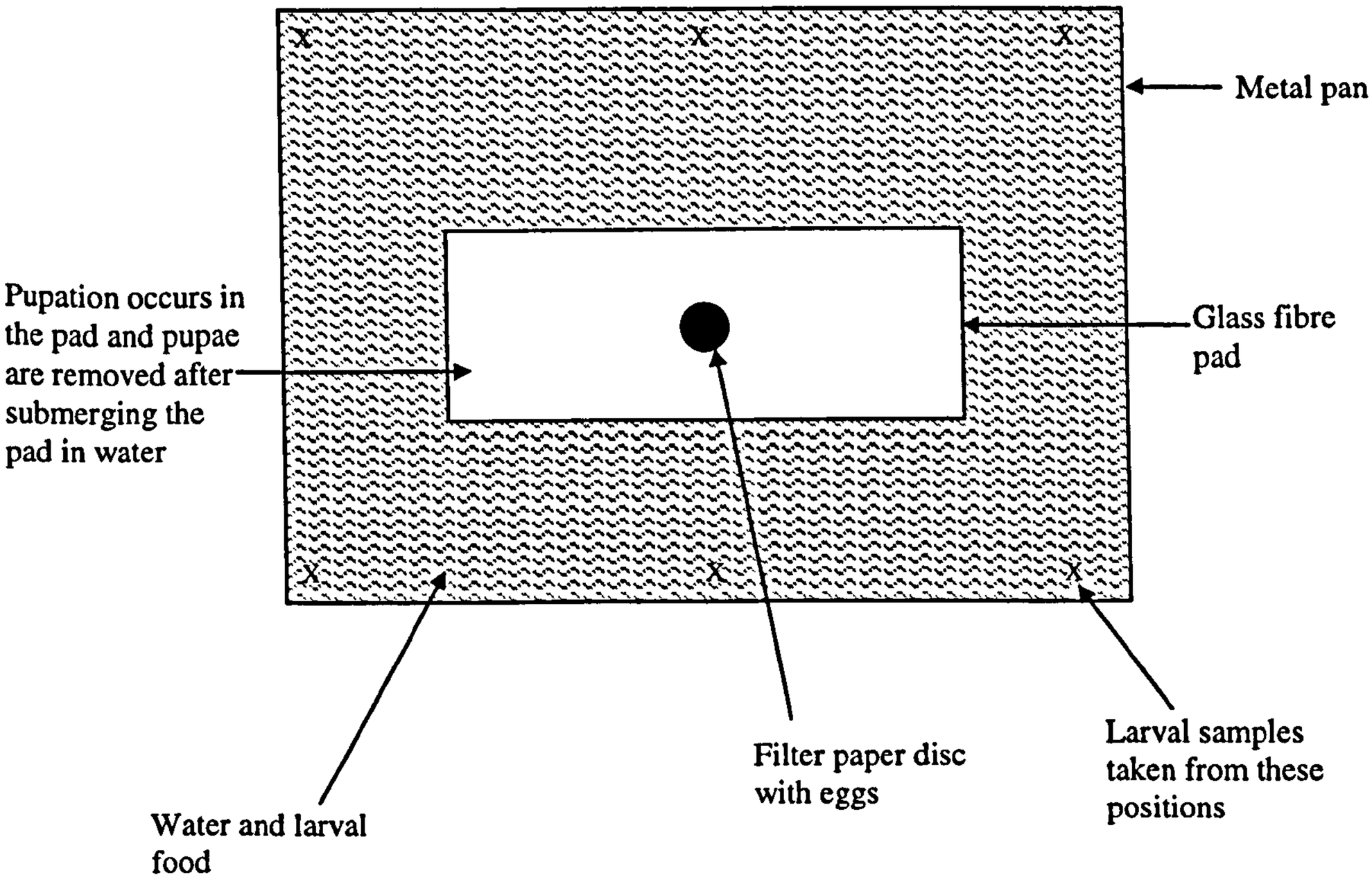
Development rate trials

Rearing conditions: Eggs and larvae were reared in a metal pan (40 x 27 x 5 cm) containing a glass fibre pad (32 x 10 x 2 cm) and approximately 2.2 l of dechlorinated tap water (Figure 2.1a). A filter paper disc covered with eggs was placed on the pad and newly hatched larvae moved into the water. Initially, 3 ml of nutrient broth (25 g of Oxoid No. 2 nutrient broth mixed with 1 l of distilled water), 8 g of grass meal and 50 ml of larval medium from rearing pans in use in the Pirbright colony (Boorman, 1974) were added to the water as food for the developing larvae. Subsequently, 1.5 ml of nutrient broth and 4 g of grass meal were added three times a week. The water was circulated by aeration to prevent the formation of scum and topped up to the 2.2 l level as required.

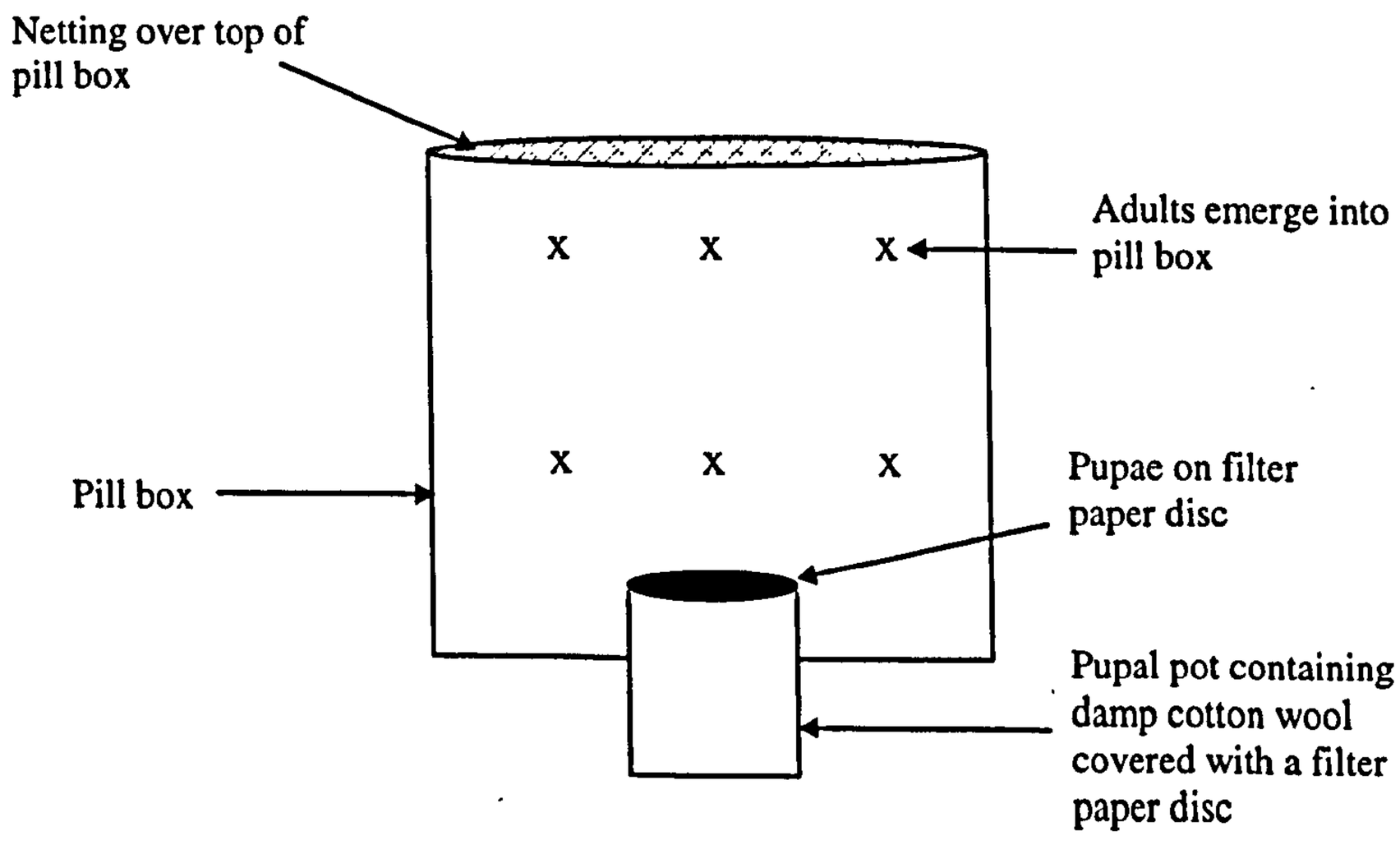
Pupae developed in the pad and were freed by flooding the pan with water. After about 15 minutes the pupae had floated to the surface and were collected in a conical flask using a suction line from a small air pump. They were then washed into a sieve and transferred to a 100 ml beaker using a gentle stream of water. The pupae floated to the surface of the water and were dispensed into pupal pots using a fine brush. The pupal pots consisted of the bottom sections of polystyrene universal tubes filled with damp cotton wool and covered with a filter paper disc. Each pot was then placed into the bottom of a waxed card pill box, into which the adults would finally emerge (Figure 2.1b).

Figure 2.1 *Culicoides* rearing conditions

a. Egg and larval rearing pans (top view)



b. Pupal pots and adult pill boxes



Experimental procedure: Adult *Culicoides* from the Pirbright colonies were allowed to lay eggs on filter paper discs, that had been divided into 30 sections. Each disc contained about 7000 eggs, that were generally evenly distributed among the sections. On the day the eggs were laid, the discs were placed with the eggs uppermost in the rearing pans at the appropriate temperature. A single filter paper disc section was then removed daily and observed under a microscope to determine the time required for the eggs to hatch. In addition, 3 ml samples of larval substrate were taken daily from six positions around the edge of the pan (Figure 2.1a), where the larvae tended to accumulate. The samples were observed under a microscope and the time required for the first emergence of the 2nd-4th larval instars was determined. Each larval instar differs markedly in size, making age characterisation straightforward.

The pad was examined to detect the formation of the first pupa, after which time the pan was flooded daily. The numbers of pupae formed on each day were counted and up to 150 (50 x 3) were placed into pupal pots. When the adults emerged the empty pupal cases (exuviae) remained in the pupal pots. To determine the duration of the pupal stage, exuviae were therefore removed from the pupal pots on a daily basis. In addition, this information was used to determine the overall time required for adult emergence. However, for days where >150 pupae formed it was necessary to extrapolate adult emergence times to take account of these additional individuals. For example, if 50 and 100 pupae from the sample of 150 eclosed on days 44 and 45 respectively and the total number of pupae collected was 2000 then actually 667 and 1333 individuals would have emerged on these days. At 15 and 30°C all the *C. variipennis sonorensis* pupae produced were kept until eclosion and the number of males and females that emerged were counted.

Finally, the wing lengths of 30 male and female *C. nubeculosus* and *C. variipennis sonorensis* that had developed at each of the temperatures were measured, from the basal arculus to the tip, using an eye piece graticule. Akey *et al.* (1978) found that the wing lengths of *C. variipennis sonorensis* were directly proportional to dry weight. Consequently in this study, wing length was used as a measure of body size.

Data analysis: Development rate curves for time to mean adult emergence were generated for each *Culicoides* species by linear regression of development rates

(1/days) against temperature. The theoretical minimum temperature for development (i.e. temperature at which development can no longer occur) was estimated by extrapolation of the regression lines to the x-axis (i.e. where development rate = 0). The number of day degrees above the minimum temperature that were required for development was calculated from the reciprocal of the slope of the lines. The regression lines for the two species were compared using analysis of covariance (ANCOVA).

G-tests were used to assess whether the sex ratio of *C. variipennis sonorensis* adults reared at 15 and 30°C was significantly different from 1:1. The emergence times of male and female *C. variipennis sonorensis* reared at 15 and 30°C were compared using Kolmogorov-Smirnov two-sample tests. In addition, to determine whether the pattern of female emergence was similar at the two rearing temperatures, the proportion of females that emerged within the first, middle and last third of the total emergence time were compared between the temperatures using a 1-way analysis of variance (ANOVA) with binomial errors (for proportion data; Crawley, 1993).

Wing lengths of male and female *C. nubeculosus* and *C. variipennis sonorensis* were regressed as linear functions of temperature. The regression lines for males and females of the same species were compared using ANCOVA.

Immature survivorship trials

Rearing conditions: Eggs and larvae were reared in a small metal pan (21 x 16 x 5 cm) containing a glass fibre pad (16 x 6 x 2 cm) and 700 ml of dechlorinated tap water. Initially, 1 ml of nutrient broth, 4 g of grass meal and 17 ml of larval medium from rearing pans in use in the Pirbright colony were added. Subsequently, 0.5 ml of nutrient broth and 2 g of grass meal were added three days a week. The rest of the rearing procedure was the same as for the development rate trials.

Experimental procedure: One thousand eggs, obtained from the Pirbright colony on the day they were laid, were counted on to a filter paper disc and placed into a rearing pan at the appropriate temperature. The numbers of pupae and adults that subsequently developed were counted. Three replicates were carried out at each temperature.

Egg hatching trials were also conducted. One hundred eggs were counted on to a filter paper disc and then placed into a rearing pan at the appropriate temperature. The number of eggs that had hatched after three weeks at 12.5 and 15°C, after two weeks at 20 and 25°C and after one week at 30 and 35°C were counted. Five replicates were carried out at each temperature.

Data analysis: The proportion of immature *Culicoides* that survived to adulthood was transformed using the arcsine square root transformation and then regressed against temperature. The information obtained from the trials was also used to determine egg, larval and pupal survivorship at the different temperatures. For example, the number of eggs that hatched in the 100 egg sample was extrapolated to give the number of larvae that would be expected to develop from 1000 eggs. Larval survivorship was then calculated by subtracting the number of pupae from the initial number of larvae. Similarly pupal survivorship was calculated by subtracting the number of adults that emerged from the number of pupae.

Effect of rearing temperature on adult survival

Culicoides variipennis sonorensis were reared to adulthood at 15, 25 and 35°C (see rearing conditions for development rate trials). One day old adults were then allowed to blood-feed on an anaesthetised mouse for about one hour, according to the method of Boorman (1974). After this time the midges were lightly anaesthetised with carbon dioxide and fully engorged females were separated from midges that had not fed. Engorged females were placed into pill boxes (about 20 individuals/box), which had fine mesh tops and a moistened filter paper disc in the base to provide a site for oviposition, and kept at 15, 25 or 35°C. *Culicoides* (in common with most insects) are susceptible to desiccation. As warmer air can hold more water vapour than air at lower temperatures, increasing temperature has the effect of reducing relative humidity for a given amount of moisture in the atmosphere. To avoid the potential confounding effect of differences in relative humidity at the different maintenance temperatures, the pill boxes were placed in exsiccators, where the humidity was maintained at 75% for all temperatures using a saturated solution of NaCl (Winston and Bates, 1960). However, it must be borne in mind that saturation deficit, a measure of the drying power of air based on both humidity and temperature, increases with temperature. A

pad of cotton wool soaked in 10% sucrose solution was placed on the mesh top of the pill boxes for one hour a day. This enabled the midges to feed but did not alter the humidity too greatly. The number of midges that died each day was then counted until all the midges had died and their wing lengths were measured.

Survival analysis with exponential errors (to describe a Type II survivorship curve where the risk of death is independent of age) and a reciprocal link was carried out on the times to death, to determine the mean survival times and daily survival rates. The effect of rearing temperature and adult maintenance temperature on survival was assessed using a 2-way ANOVA. The influence of rearing temperature on wing length was determined using a 1-way ANOVA with normal errors.

2.4 Results

Development rate trials

The development times for larval, pupal and adult stages of *C. nubeculosus* and *C. variipennis sonorensis* reared at different temperatures are shown in Table 2.1. The mean time to adult emergence was greater at low temperatures and the range of time over which adults emerged was prolonged.

The development rate (1/development time) of *C. nubeculosus* ($F_{1,4} = 200.15$, $p < 0.001$) and *C. variipennis sonorensis* ($F_{1,4} = 131.18$, $p < 0.001$) increased linearly between 12.5 and 35°C (Figure 2.2). The theoretical minimum temperature for development was estimated to be 8.1°C for *C. nubeculosus* and 10.7°C for *C. variipennis sonorensis*. The total number of day degrees required above the minimum temperature for development was estimated to be 416.7 for *C. nubeculosus* and 333.3 for *C. variipennis sonorensis*. Although the slopes of the development rate curves did not differ significantly between the two species ($F_{1,8} = 3.9$, NS), the development rate of *C. nubeculosus* was faster than that for *C. variipennis sonorensis* at low temperatures, while at high temperatures the reverse was true.

Table 2.1 Development times for *C. nubeculosus* and *C. variipennis sonorensis* reared at different temperatures.

a. *C. nubeculosus*

Temperature °C	Day of first emergence of larval stages				Day of pupal formation range and mean ± SE	Pupal duration (days) mean ± SE	Day of adult emergence range and mean ± SE
	*1st instar	2nd instar	3rd instar	4th instar			
12.5	10	12	26	36	74 – 131 97.2 ± 0.17	9.2 ± 0.01	80 – 140 **106.3 ± 0.18**
15	6	8	16	22	41 – 83 52.0 ± 0.07	6.8 ± 0.01	46 – 88 **58.8 ± 0.07**
20	4	6	10	12	29 – 42 32.4 ± 0.12	3.7 ± 0.03	32 – 45 **36.1 ± 0.12**
25	2	4	7	9	15 – 30 20.8 ± 0.03	2.4 ± 0.02	17 – 32 **23.3 ± 0.07**
30	2	3	5	7	14 – 29 19.5 ± 0.03	1.7 ± 0.01	15 – 30 **21.1 ± 0.08**
35	1	2	4	6	11 – 16 13.3 ± 0.17	1.9 ± 0.06	12 – 17 **15.1 ± 0.19**

b. *C. variipennis sonorensis*

Temperature °C	Day of first emergence of larval stages				Day of pupal formation range and mean ± SE	Pupal duration (days) mean ± SE	Day of adult emergence range and mean ± SE
	*1st instar	2nd instar	3rd instar	4th instar			
12.5	11	12	40	53	94 – 184 132.2 ± 0.5	9.2 ± 0.02	102 – 193 **142.2 ± 0.59**
15	5	7	21	25	45 – 106 70.6 ± 0.13	7.3 ± 0.01	51 – 113 **77.2 ± 0.14**
20	4	6	7	9	25 – 51 35.1 ± 0.06	3.4 ± 0.01	28 – 54 **38.3 ± 0.06**
25	2	4	6	9	16 – 33 24.4 ± 0.07	2.2 ± 0.01	18 – 35 **26.5 ± 0.08**
30	2	3	5	7	9 – 24 13.7 ± 0.02	1.5 ± 0.01	10 – 25 **15.2 ± 0.02**
35	1	2	3	4	8 – 21 12.9 ± 0.04	1.6 ± 0.02	9 – 22 **14.3 ± 0.04**

*equivalent to time required for first eggs to hatch

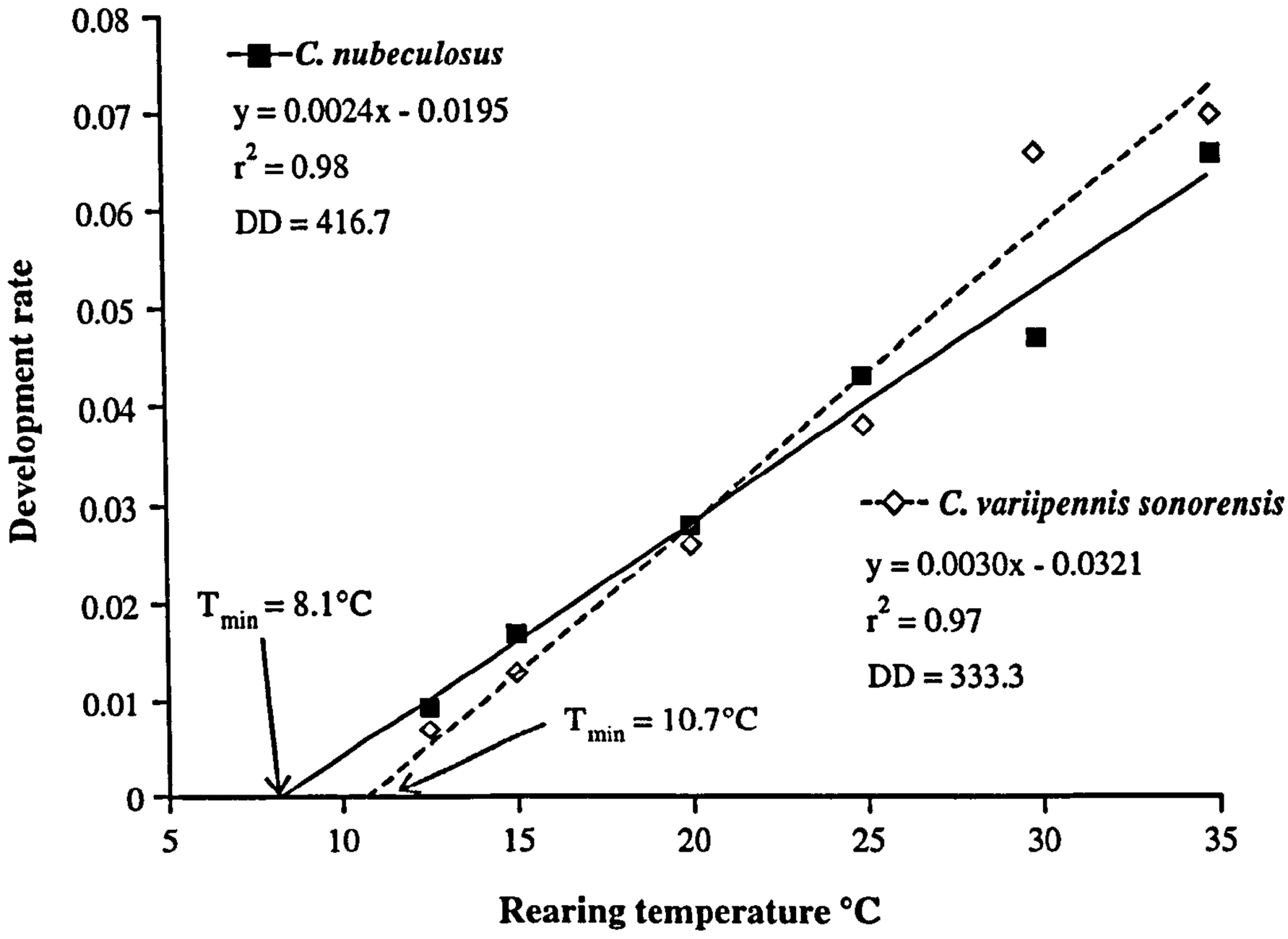
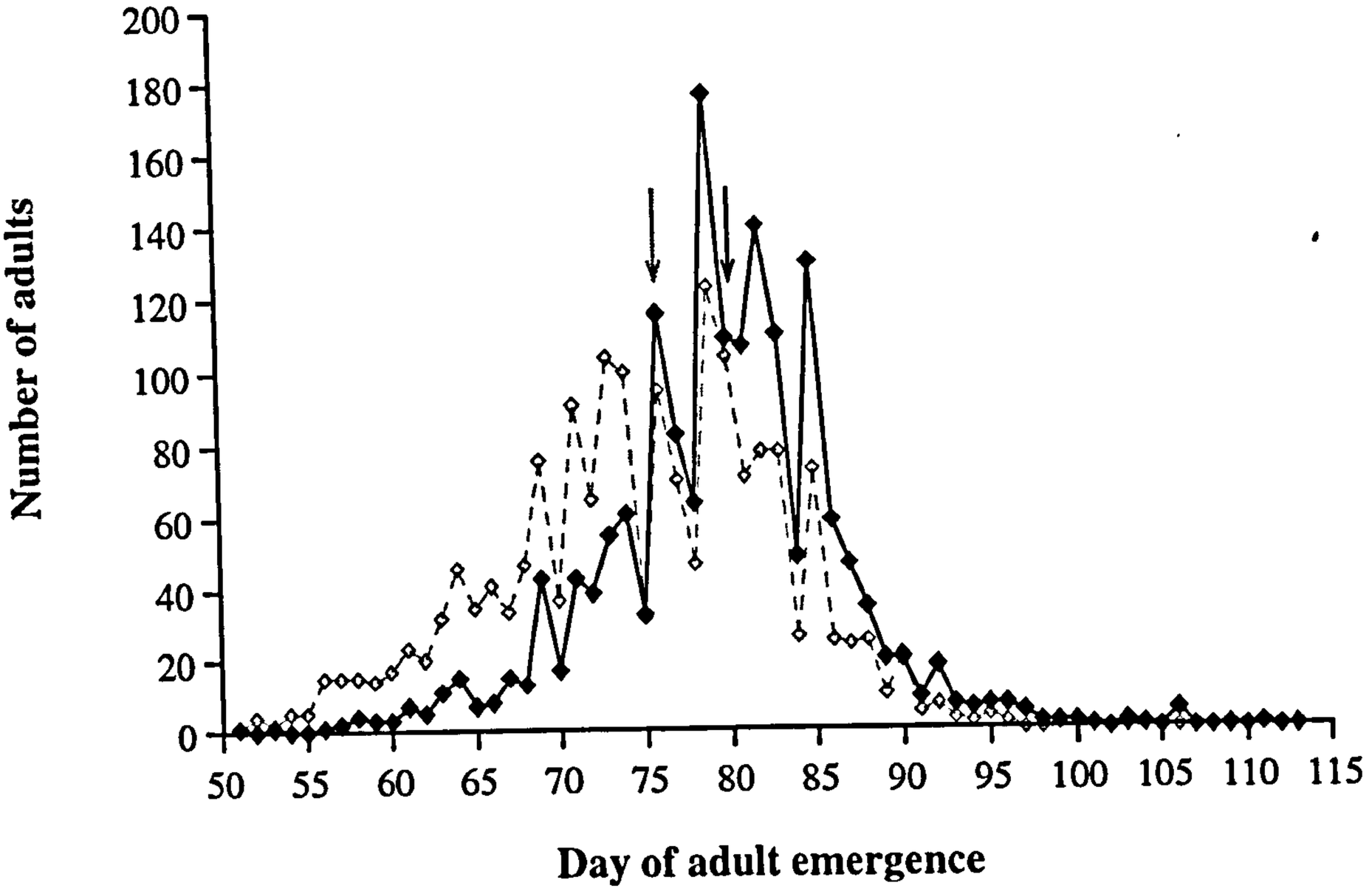


Figure 2.2 Development rates (1/mean development time) to adulthood for *C. nubeculosus* and *C. variipennis sonorensis* reared at different temperatures. T_{min} is the theoretical minimum temperature for development and DD is the total number of day degrees required above T_{min} for development.

The sex ratio of *C. variipennis sonorensis* reared at 15°C (G = 0.6, df = 1, NS) or 30°C (G = 3.27, df = 1, NS) did not significantly differ from 1:1. However, males tended to emerge before the females at both 15 (Kolmogorov-Smirnov two-sample test, $p < 0.001$) and 30°C (Kolmogorov-Smirnov two-sample test, $p < 0.001$) (Figure 2.3). In addition, the pattern of female emergence differed between the two rearing temperatures (Table 2.2). For example, a significantly greater proportion of females emerged within the first third of the total emergence time at rearing temperatures of 30°C compared to 15°C, while for the middle third of the total emergence time, the reverse was true (Table 2.2).

a. 15°C



b. 30°C

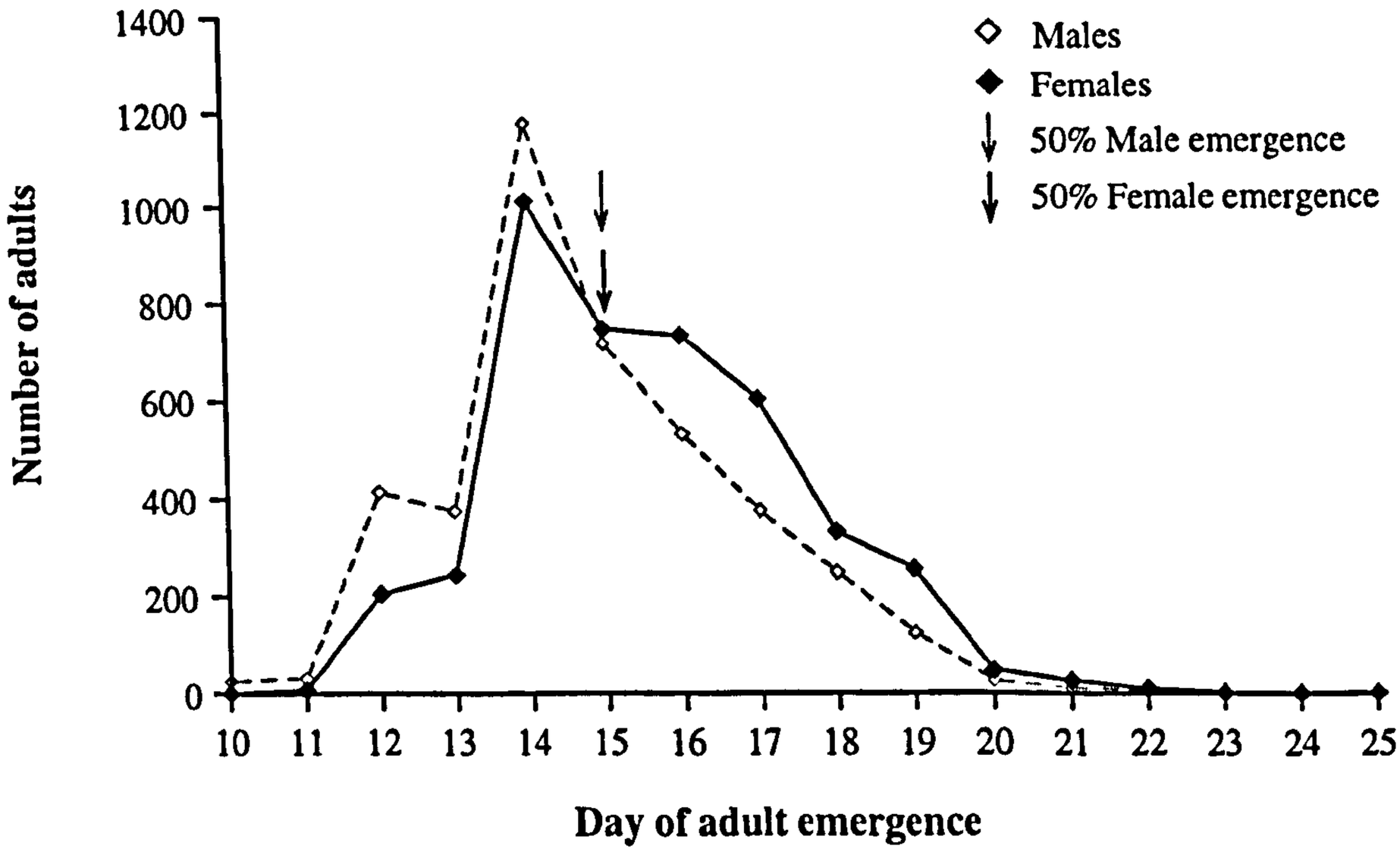


Figure 2.3 Emergence times for male and female *C. variipennis sonorensis* reared at (a) 15°C and (b) 30°C.

Table 2.2 Results of statistical analyses comparing the proportion of female *C. variipennis sonorensis* that emerged within the first, middle and last third of the total emergence time, at rearing temperatures of 15 and 30°C.

Emergence time	Proportion of females to emerge (days of emergence)		χ^2 df = 1
	15°C	30°C	
First third	0.12 (days 51-71)	0.35 (days 10-14)	370.3 $p < 0.001$
Middle third	0.86 (days 72-92)	0.63 (days 15-19)	325.2 $p < 0.001$
Last third	0.02 (days 93-113)	0.02 (days 20-25)	2.6 NS

The wing length of male ($F_{1,4} = 12.38$, $p < 0.05$) and female ($F_{1,4} = 13.83$, $p < 0.05$) *C. nubeculosus* and male ($F_{1,4} = 705.42$, $p < 0.001$) and female ($F_{1,4} = 220.37$, $p < 0.001$) *C. variipennis sonorensis* was inversely related to rearing temperature between 12.5 and 35°C (Figure 2.4). Female wing lengths were larger than male wing lengths for *C. variipennis sonorensis* ($F_{1,9} = 120.63$, $p < 0.001$) and *C. nubeculosus*, although for the latter this difference was not significant ($F_{1,9} = 3.52$, NS). The impact of rearing temperature on wing length did not vary significantly between male and female *C. nubeculosus* ($F_{1,8} = 0.01$, NS) or male and female *C. variipennis sonorensis* ($F_{1,8} = 3.87$, NS).

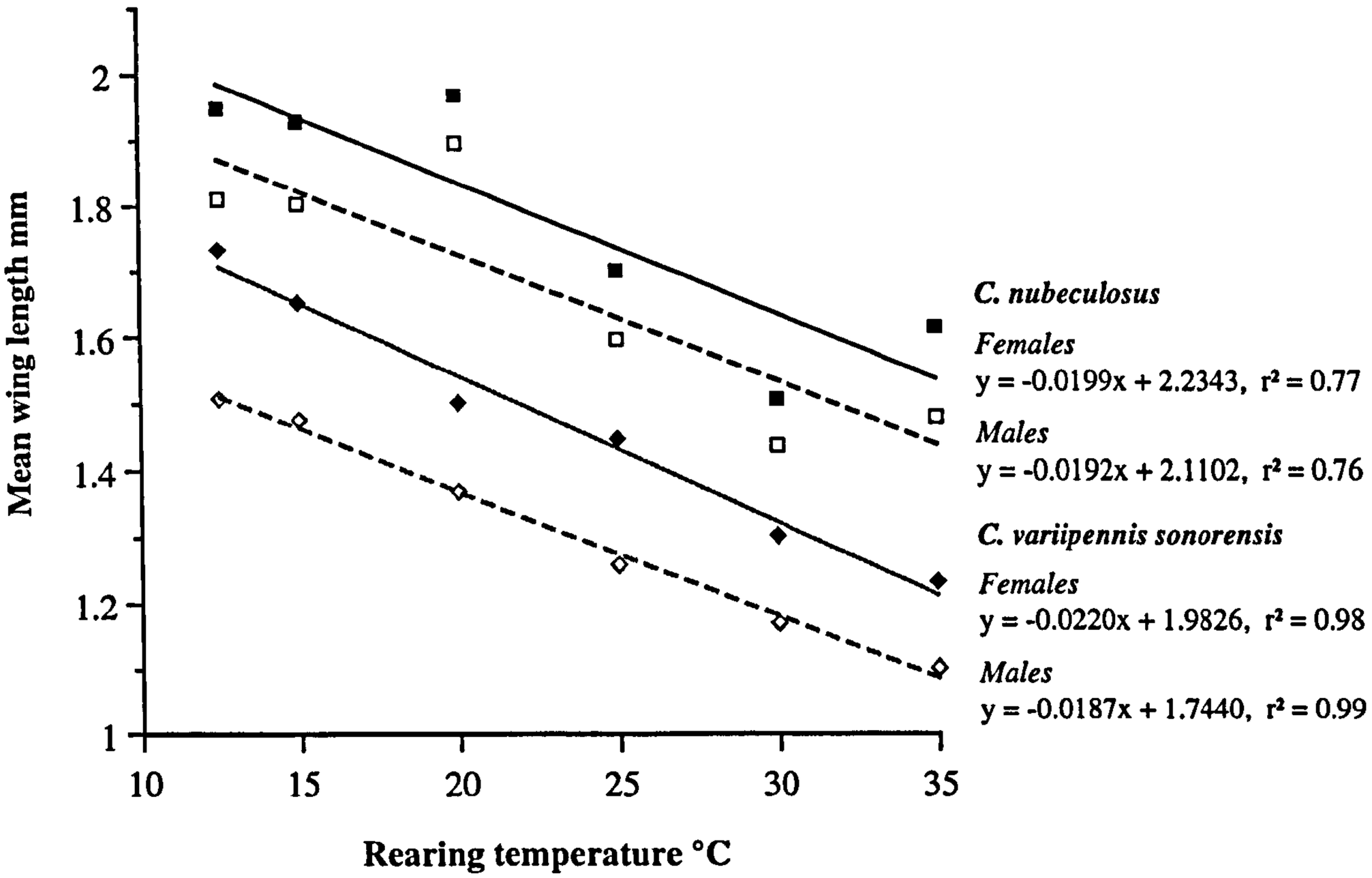


Figure 2.4 Mean wing lengths of male (open squares) and female (solid squares) *C. nubeculosus* and male (open diamond) and female (solid diamonds) *C. variipennis sonorensis* reared at different temperatures.

Immature survivorship

The survivorship of both *Culicoides* species to adulthood was affected by rearing temperature (Figure 2.5). Maximum survivorship to adulthood occurred at rearing temperatures of 25 and 30°C for *C. nubeculosus* and at 25°C for *C. variipennis sonorensis*. At low rearing temperatures (12.5-15°C) survivorship of *C. variipennis sonorensis* was severely reduced compared to that for *C. nubeculosus*, while at very high temperatures (35°C) the reverse was true. The equations describing the relationship between the arcsine square root of the proportion of immatures surviving to adulthood (y) and temperature (x) are:

C. nubeculosus

12.5-30°C: $y = 1.189x + 17.238$, $r^2 = 0.79$, $F_{1,13} = 48.42$, $p < 0.001$

30-35°C: $y = -8.011x + 292.798$

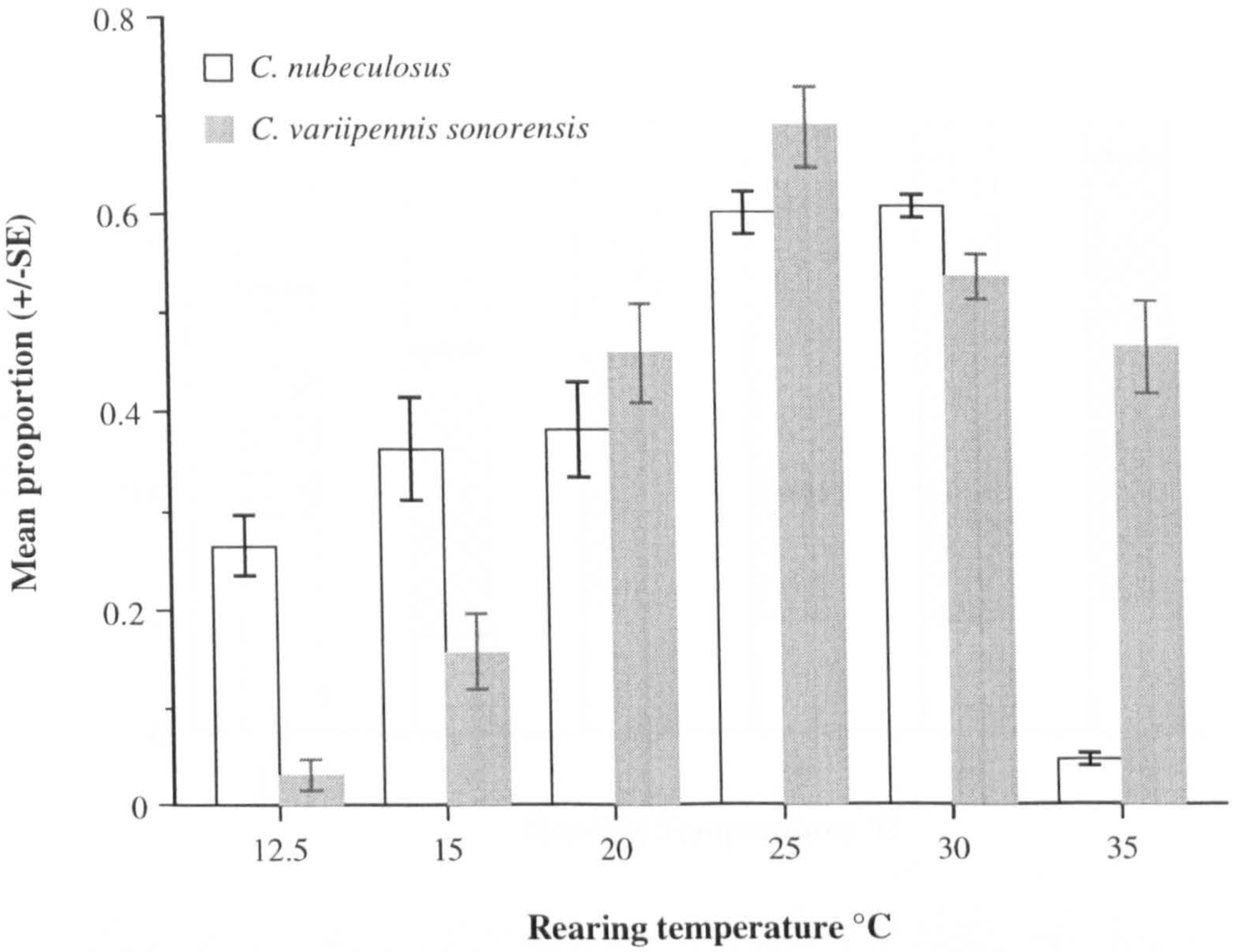


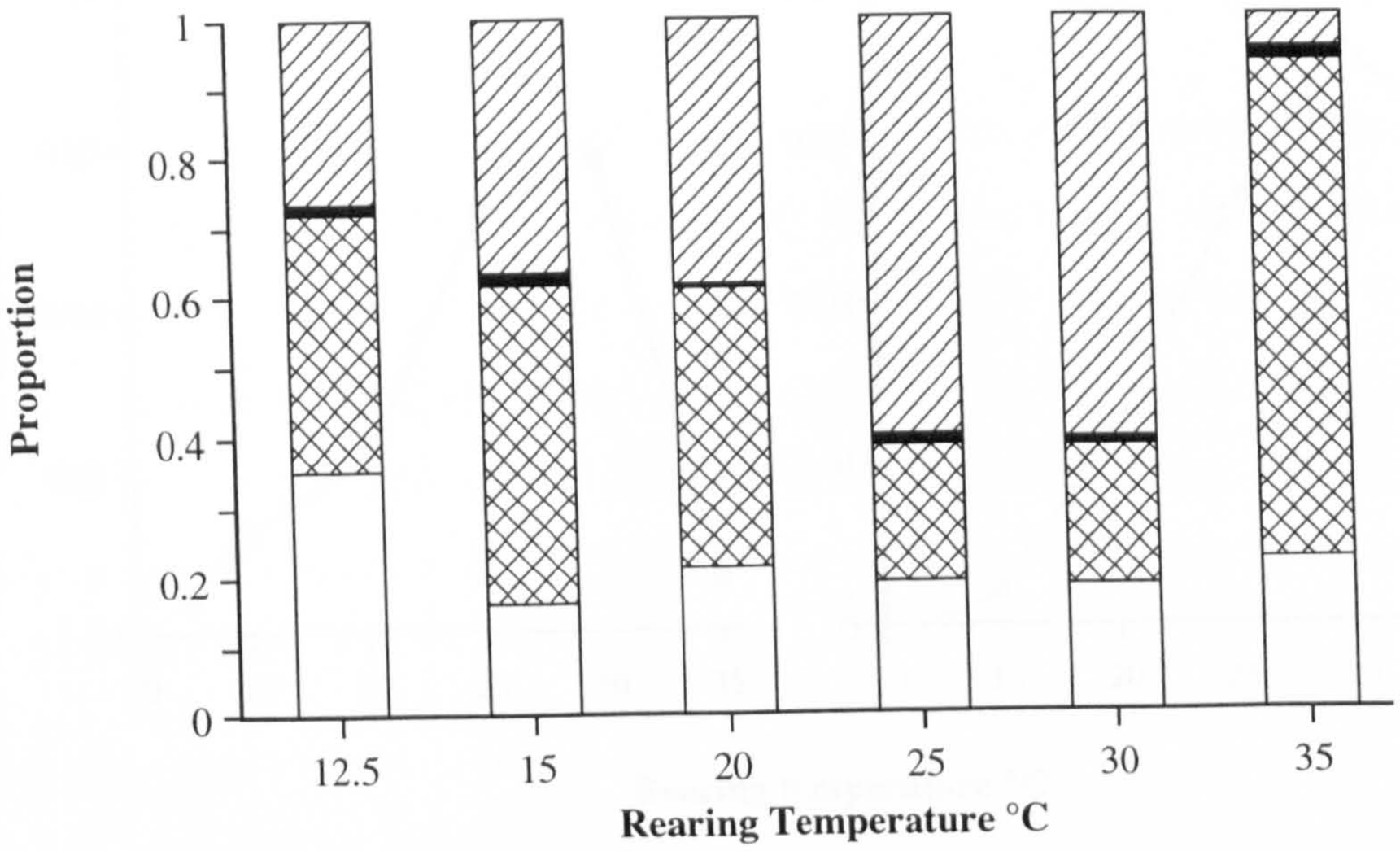
Figure 2.5 Proportion of immature *C. nubeculosus* and *C. variipennis sonorensis* that survived to adulthood when reared at different temperatures.

C. variipennis sonorensis

12.5-35°C: $y = -0.196x^2 + 10.723x - 93.172$, $r^2 = 0.90$, $F_{2,17} = 78.50$, $p < 0.001$

In general, immature mortality was greatest in the egg and larval stages (Figure 2.6).

a. *C. nubeculosus*



b. *C. variipennis sonorensis*

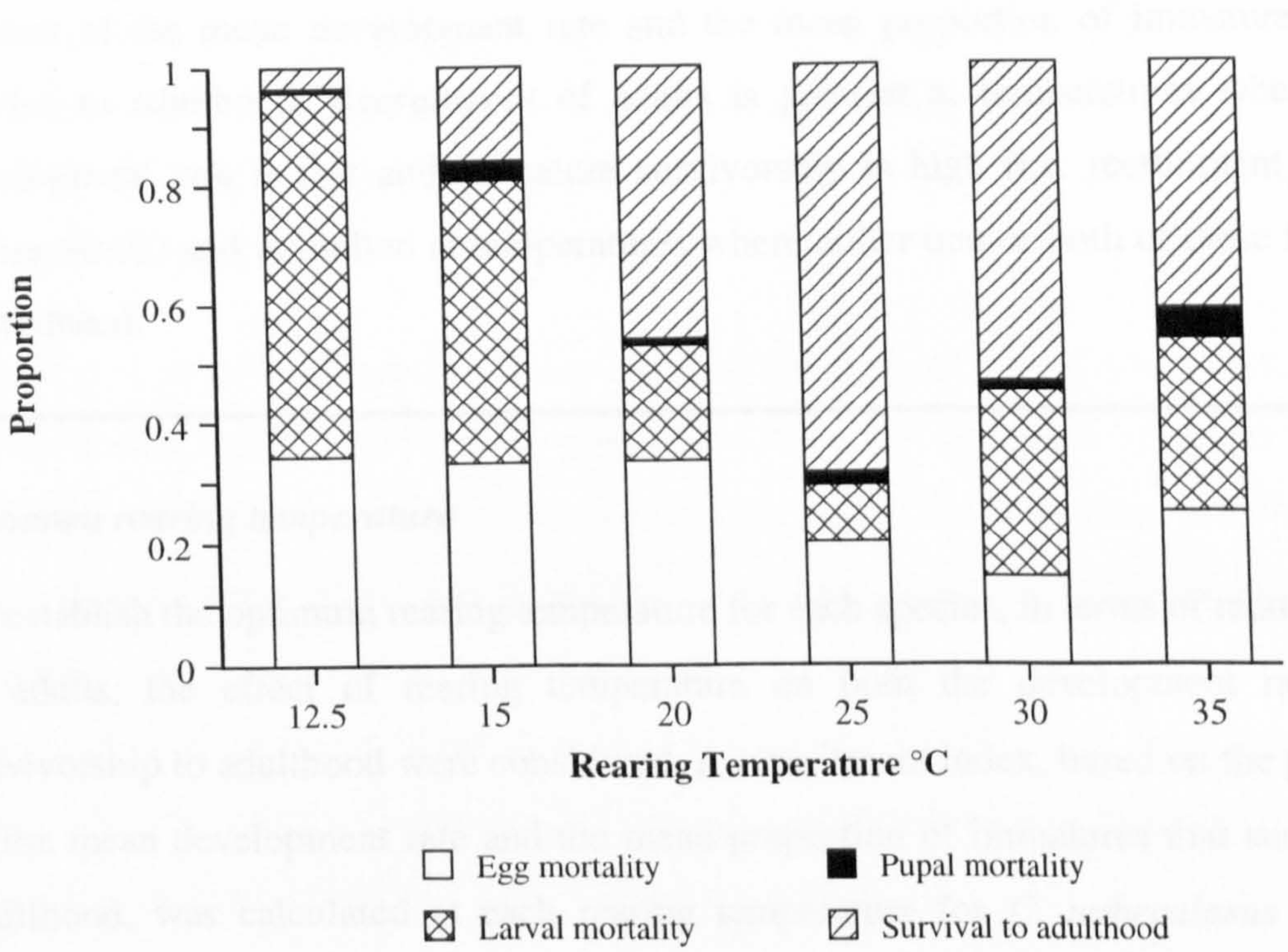


Figure 2.6 Immature mortality for (a) *C. nubeculosus* and (b) *C. variipennis sonorensis* reared at different temperatures.

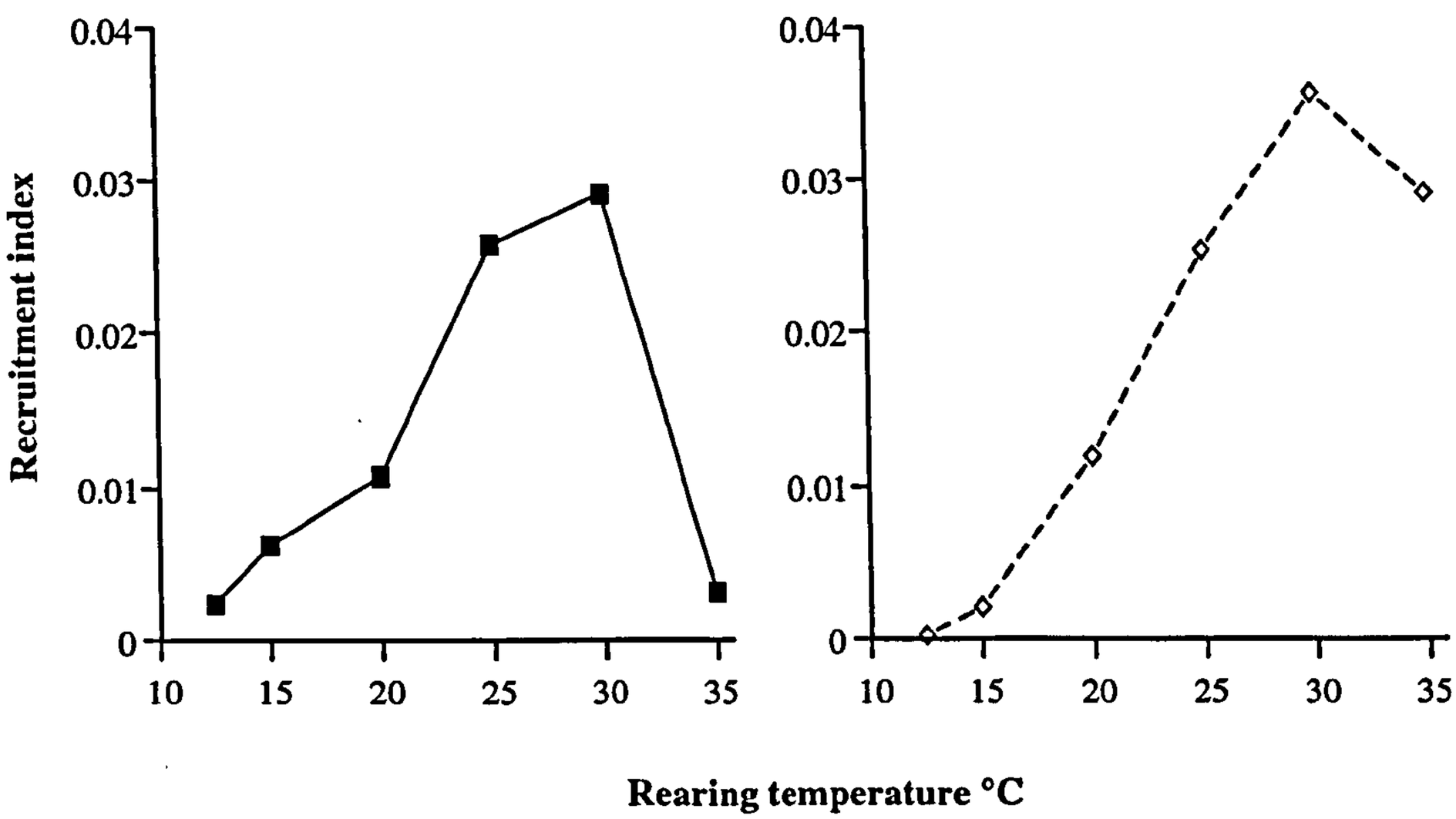


Figure 2.7 Effect of rearing temperature on recruitment of adults from the immature stages for *C. nubeculosus* and *C. variipennis sonorensis*. The recruitment index is the product of the mean development rate and the mean proportion of immatures that survive to adulthood. Recruitment of adults is greatest at temperatures where the development rate is fast and immature survivorship is high (i.e. recruitment index values >0.02) and is limited at temperatures where either one or both of these factors are reduced.

Optimum rearing temperature

To establish the optimum rearing temperature for each species, in terms of recruitment of adults, the effect of rearing temperature on both the development rate and survivorship to adulthood were considered. A recruitment index, based on the product of the mean development rate and the mean proportion of immatures that survive to adulthood, was calculated at each rearing temperature for *C. nubeculosus* and *C. variipennis sonorensis* (Figure 2.7). The recruitment index was greatest at rearing temperatures of 25 to 30°C for *C. nubeculosus* and at 25 to 35°C for *C. variipennis sonorensis* (Figure 2.7). These temperature ranges were therefore considered to be the optimum rearing temperatures. At temperatures below the optimum, recruitment was

limited due to the extended development time and poor survivorship, while at temperatures above the optimum recruitment was limited due to low survivorship.

Effect of rearing temperature on adult survival

Survival of female *C. variipennis sonorensis* was not affected by rearing temperature (χ^2 = 1.62, df = 2, NS), but was significantly influenced by the adult maintenance temperature (χ^2 = 107.5, df = 2, $p < 0.001$) (Table 2.3). The interaction between rearing temperature and adult maintenance temperature was not significant (χ^2 = 6.22, df = 4, NS). Wing length was affected by rearing temperature ($F_{2,311}$ = 1053.4, $p <$ 0.01) and females reared at low temperatures were larger than those reared at higher temperatures (Table 2.3).

2.5 Discussion

Culicoides nubeculosus development from egg to adult can occur at temperatures ranging from ≈8.1°C to 35°C, while *C. variipennis sonorensis* development can occur from ≈10.7 to at least 35°C and probably at even higher temperatures. The optimum temperature range for development was 25-30°C for *C. nubeculosus* and 25-35°C for *C. variipennis sonorensis*. Temperatures experienced during the immature stages did not affect survival of adult *C. variipennis sonorensis*, when adults were maintained at a range of temperatures.

Rearing temperature affected both immature survivorship and the development rate of *C. nubeculosus* and *C. variipennis sonorensis*. For example, at the optimum rearing temperatures, survivorship to adulthood was high and the mean development time and range of time over which adults emerged was short. Large numbers of adults are therefore likely to be rapidly recruited at these temperatures. Since outbreaks of BT and AHS tend to occur when vectors are abundant (Mellor and Boorman, 1995; Mellor, 1996), risk of an outbreak (following the introduction of virus) will be high at times of year when conditions are optimal for development.

Low temperatures adversely affected development in both species with survivorship to the adult stage reduced and the mean time and range of adult emergence prolonged. In addition, the emergence of females with regards to the total emergence time was delayed at low rearing temperatures. Since BTV and AHSV are transmitted by female

Adult temperature °C	Immature temperature °C	Sample size	Wing length mm ± SE	Survival range days	Mean survival days	+SE	-SE	Survival rate / day	Overall mean survival days (+/- SE)	Overall survival rate / day
15	15	32	1.67 ± 0.01	10 - 38	26.1	5.6	3.9	0.96	**22** (2.3/1.9)	0.96
	25	48	1.45 ± 0.01	3 - 38	20.0	1.4	1.1	0.95		
	35	32	1.21 ± 0.01	2 - 50	20.8	2.0	1.6	0.95		
25	15	33	1.63 ± 0.01	3 - 12	7.8	1.6	1.2	0.88	**7.8** (0.6/0.5)	0.88
	25	51	1.45 ± 0.01	3 - 18	9.0	0.4	0.4	0.89		
	35	23	1.26 ± 0.02	3 - 11	5.3	0.7	0.6	0.83		
35	15	28	1.67 ± 0.01	3 - 8	5.4	1.3	0.9	0.83	**5.8** (0.5/0.4)	0.84
	25	47	1.45 ± 0.01	3 - 14	6.6	0.3	0.2	0.89		
	35	20	1.26 ± 0.01	2 - 10	4.5	0.6	0.5	0.80		

Table 2.3 Survival of blood–fed female *C. variipennis sonorensis*, reared as immatures and maintained as adults, at different temperatures. Humidity was maintained at 75%. Females were 1 day old at the time of the blood meal.

Culicoides, this delay combined with the above factors could result in a reduced rate of viral transmission at low temperatures. Nevertheless, it was essential to establish the minimum temperature for development in both species, as this can be used to assess whether development and hence adult *Culicoides* could occur throughout the year in a particular region. This is important because the continual presence of adults could result in the viruses becoming endemic (Mellor, 1993, 1994).

Although the optimum rearing temperatures for *C. nubeculosus* and *C. variipennis sonorensis* overlapped, the two species responded differently to low and very high temperatures. For example, at low temperatures (≤15°C) *C. nubeculosus* was more successful than *C. variipennis sonorensis*, with comparatively shorter developmental periods and more individuals surviving to adulthood. However at 35°C, the reverse was true and while 35°C was within the optimum range for *C. variipennis sonorensis* development, immature survivorship of *C. nubeculosus* was severely reduced.

The number of adults in a population will be influenced by the survival rate of adult midges, as well as recruitment from the developing immatures. Small body size has been correlated with a reduction in survival in other insect species (e.g. McCabe and Partridge, 1997). However, although rearing temperature affected *Culicoides* body size, it did not significantly influence adult survival when adult *C. variipennis sonorensis* were maintained at a range of temperatures. Consequently, the main impact of rearing temperature on population size appears to be through its effects on the development rate and survivorship of the immatures. However, population size may also be indirectly influenced by factors not considered here. For example, given that body size and temperature are inversely related, further trials are necessary to determine whether rearing temperature affects the desiccation tolerance of adult midges (as smaller individuals may be more susceptible to desiccation) or fecundity (smaller females lay fewer eggs per batch; Akey *et al.*, 1978).

The present trials involving *C. variipennis sonorensis* overcame some of the drawbacks associated with previous work in this area. In the trials by Vaughan and Turner (1987), *C. variipennis australis* (= *C. variipennis sonorensis*; Tabachnick, 1996) larvae were nutritionally stressed, resulting in prolonged development and poor immature survivorship. Here, I reared the larvae under optimum conditions. In

addition, Mullens and Rutz (1983) reported the effect of temperature on the development of a New York population of *C. variipennis*. However, since *C. variipennis sonorensis* does not occur as far north as New York, it is likely that this was another subspecies within the *C. variipennis* complex, *C. variipennis variipennis*. *Culicoides variipennis variipennis* is found in cooler northern regions of the USA and hence is not as good a model as *C. variipennis sonorensis* for establishing the influence of temperature on development of *C. imicola*.

If the impact of temperature on *C. imicola* is similar to that for *C. variipennis sonorensis*, it is likely that *C. imicola* will be most successful in the warmer areas of its range in Europe and at times of year when conditions are hottest. There is also concern that the spread of *C. imicola* across Europe may not be complete (e.g. Rawlings *et al.*, 1998) and that if conditions should warm (as predicted by climate change scenarios; Intergovernmental Panel on Climate Change, 1996) this extension in range could be even greater. Currently there is no evidence to suggest that *C. imicola* diapause as fourth instar larvae during winter (Mellor, pers. comm.), which is the typical strategy of Palearctic *Culicoides* species. If this is the case, then the distribution of *C. imicola* is likely to be restricted to areas where the temperature is above the lower developmental threshold for the majority of the year. Consequently, the minimum temperature for development established in the model species *C. variipennis sonorensis* may provide some insight into the areas where *C. imicola* could occur, both currently and as conditions warm. This is investigated further in Chapter 6.

In addition to changes in the distribution of *C. imicola*, climate change could affect the number of generations that *Culicoides* species undergo in a year. For example, according to the method proposed by Yamamura and Kiritani (1998) for estimating the potential increase in number of generations under global warming in temperate zones, *C. nubeculosus* and *C. variipennis sonorensis* would be able to complete at least one more generation per year. An increase in the number of generations would result in a greater number of adult *Culicoides* vectors and hence enhance the likelihood of a BT or AHS epidemic, following the introduction of virus. In addition, due to the predicted extension in the development season (i.e. spring is expected to arrive earlier and autumn later; Climate Change Impacts Review Group, 1996; Menzel

and Fabian, 1999) *Culicoides* development and hence adult vectors could potentially occur over a greater proportion of the year. This could result in areas of Europe experiencing outbreaks of BT or AHS over several successive seasons, where previously outbreaks would have finished during winter of the first season.

In this chapter I have investigated the effect of temperature on the development of *C. nubeculosus* and *C. variipennis sonorensis*. By using these species as models for the vector species that occur in Europe (e.g. *C. obsoletus*, *C. pulicaris* and *C. imicola*), the information established in this study may be used to help identify periods of risk for virus transmission in Europe. In addition, this study has provided useful insight to some of the ways in which climate change may affect *Culicoides* vectors and their potential for virus transmission.

CHAPTER 3: EFFECT OF TEMPERATURE ON THE TRANSMISSION OF ORBIVIRUSES BY *CULICOIDES* BITING MIDGES (DIPTERA: CERATOPOGONIDAE): THE *C. VARIIPENNIS SONORENSIS* MODEL.

3.1 ABSTRACT

The influence of temperature on the likelihood of *Culicoides variipennis sonorensis* transmitting African horse sickness virus (AHSV) serotypes 4 and 6, bluetongue virus (BTV) serotypes 10 and 16 and epizootic haemorrhagic disease virus (EHDV) serotype 1 was investigated. Extrinsic incubation periods (EIP), vector competence and vector survival were determined at 15, 20, 25 and 30°C. The impact of moisture levels on vector survival was also investigated by maintaining adults at 40, 75 and 85% relative humidity at each temperature. Higher temperatures were associated with a shorter EIP for all virus serotypes except AHSV6 to which *C. variipennis sonorensis* was refractory, increased vector competence for AHSV4 and EHDV1, but not for BTV10 or BTV16, and a reduction in vector survival. Humidity interacted with temperature in influencing vector survival, such that at low temperatures lower humidity (40 and 75% RH) was detrimental for survival, while at high temperatures high humidity (85% RH) was detrimental. In general, the transmission potential of *C. variipennis sonorensis* for AHSV4, EHDV1, BTV10 and BTV16 was greater at higher temperatures, because although vector survival declined, this was more than compensated for by the accompanying decrease in EIP.

3.2 INTRODUCTION

Culicoides biting midges are the principal vectors of nine species of orbivirus (Calisher and Mertens, 1998), including African horse sickness virus (AHSV), which infects equids, and bluetongue virus (BTV) and epizootic haemorrhagic disease virus (EHDV), which infect ruminants. The ability of *Culicoides* to transmit these viruses is dependent on the species and genotype of the midge and is also influenced by environmental factors such as temperature. While progress has been made in identifying the genetic mechanisms controlling the competence of *Culicoides* to orbiviruses (Tabachnick, 1991), information concerning the quantitative effects of temperature on virus transmission is lacking. However, in order to identify regions of the World that are likely to be at risk of disease and enable the more effective

targeting of control measures, it is essential to have a better understanding of the relationship between temperature and virus transmission by *Culicoides*.

Temperature can affect transmission by influencing the proportion of midges that can transmit virus. This is due to its impact on vector competence, vector survival, the extrinsic incubation period (EIP; development time of the virus in the vector) and the blood-feeding interval. The relationship between these factors can be described as follows:

$M = Vp^n$ (equation 1)

where M = proportion of midges that could take at least one virus transmissive blood-meal (after initially ingesting a viraemic blood-meal), V = proportion of competent midges, p = daily survival rate and n = days to first virus transmissive blood-meal (i.e. first blood-meal after the EIP).

Although temperature has been shown to affect each of these factors, their precise relationships have not been defined. For example, with regards to vector competence, Wellby *et al.* (1996) and Mullens *et al.* (1995) found that the infection rate of *C. variipennis sonorensis* with AHSV9 or BTV11 was greater at ≥20°C, compared to incubation temperatures of ≤15°C. However, even if a midge is susceptible to infection (i.e. virus can replicate in the midgut), it does not necessarily mean that it will be competent to transmit virus, since some midges possess a midgut escape barrier (where virus is restricted to the midgut cells; Jennings and Mellor, 1987) or a dissemination barrier (where virus is unable to infect secondary target organs; Fu *et al.*, 1999). In the case of vector survival, Hunt *et al.* (1989) and Wellby *et al.* (1996) found that high temperatures reduced survivorship of *C. variipennis sonorensis*, but the effect of temperature on the daily survival rate was not determined. In addition, the relationship between temperature and duration of the EIP has not been directly quantified, although Wellby *et al.* (1996) and Mullens *et al.* (1995) found that AHSV9 and BTV11 replicated more rapidly in *C. variipennis sonorensis* at higher temperatures. The effect of temperature on the interval between blood-meals in *C. variipennis sonorensis* is the only relationship that has been established (Mullens and Holbrook, 1991).

The present study was therefore carried out to determine the influence of temperature on vector competence, vector survival and the duration of the EIP, in genetically competent *Culicoides* infected with AHSV, BTV or EHDV. In addition, since 9 AHSV serotypes, 24 BTV serotypes and 8 EHDV serotypes are currently recognised, it was necessary to establish whether the impact of temperature was consistent among serotypes. The influence of humidity on vector survivorship was also investigated, as due to their small size, adult midges are likely to be susceptible to desiccation, especially at higher temperatures (Bursell, 1964). The effect of temperature and humidity on the above parameters was determined in *C. variipennis sonorensis*, which is a competent vector of AHSV in the laboratory (Boorman *et al.*, 1975; Mellor *et al.*, 1975) and the major vector of BTV (Mellor, 1990) and EHDV (Foster *et al.*, 1977) in North America. This information was then used to quantify the effect of temperature and humidity on transmission of these orbiviruses and establish the range of conditions over which transmission could occur.

3.3 Methods

Extrinsic incubation period and vector competence

Viruses

Culicoides variipennis sonorensis were infected with five orbivirus serotypes (Table 3.1). Serotypes within a virus species were chosen on the basis of their differing distributions. All the viruses were originally obtained from Onderstepoort Veterinary Institute, South Africa. They were passaged as shown in Table 3.1 and stored as tissue culture supernatants at 4°C until required.

Insects

Two to three day old adult *C. variipennis sonorensis*, from the PIRB-s-3 strain (Wellby *et al.*, 1996) of the Pirbright colony (Boorman, 1974), were allowed to feed on a blood-virus suspension consisting of 1 ml of heparinised horse blood mixed with 1 ml of virus solution. The blood-virus suspension contained a high titre of virus (5.7-6.5 $\log_{10}$ $TCID_{50}$/ml) to overcome the 'threshold of infection' (concentration of virus required to initiate infection in a genetically competent midge). Horse blood was used as the source of blood to increase the likelihood of midges becoming infected with the viruses (Marchi *et al.*, 1995; Burroughs, unpub. obs.).

Table 3.1 Orbivirus serotypes used to infect *C. variipennis sonorensis*.
MB = suckling mouse brain, BHK = baby hamster kidney cells and E = embryonated hen eggs. The numbers by the acronyms refer to how many times the virus has been passaged in that system (e.g. MB3 BHK4 = 3 passages in a suckling mouse brain followed by 4 passages in baby hamster kidney cells).

Serotype	Passage history	Distribution
AHSV4	MB3 BHK4	Iberia 1987 – 1990, Africa
AHSV6	MB3 BHK4	Africa
BTV10	E1 BHK7	Iberia 1956 – 1960, Africa, Asia, N. America
BTV16	E2 BHK7	Africa, Asia, Australia
EHDV1	BHK11	N. America, S. America, Caribbean, Australia

Adult midges were blood-fed using the artificial feeding apparatus and method described by Mellor *et al.* (1974), except that a stretched parafilm membrane was used in place of a 1 day old chick skin membrane (Figure 3.1). They were allowed to feed for about 30 minutes, after which time they were lightly anaesthetised with carbon dioxide and the fully engorged females were separated from individuals that had not fed. Engorged females were then placed into waxed cardboard pill boxes with fine mesh tops (about 100 individuals/box). A pad of cotton wool soaked in 10% sucrose solution medicated with 100 µg/ml of penicillin/streptomycin was placed on the mesh and replaced daily. This provided a source of energy, while the presence of antibiotics in the sucrose solution has been shown to prolong survival of midges (Bellis *et al.*, 1994). A moistened filter paper disc in the base of the pill box provided sites for oviposition.

Virus-fed females were then kept at a constant temperature of either 15, 20, 25 or 30°C. At 15°C and 20°C, samples consisting of 4 pools of 5 females (to determine the

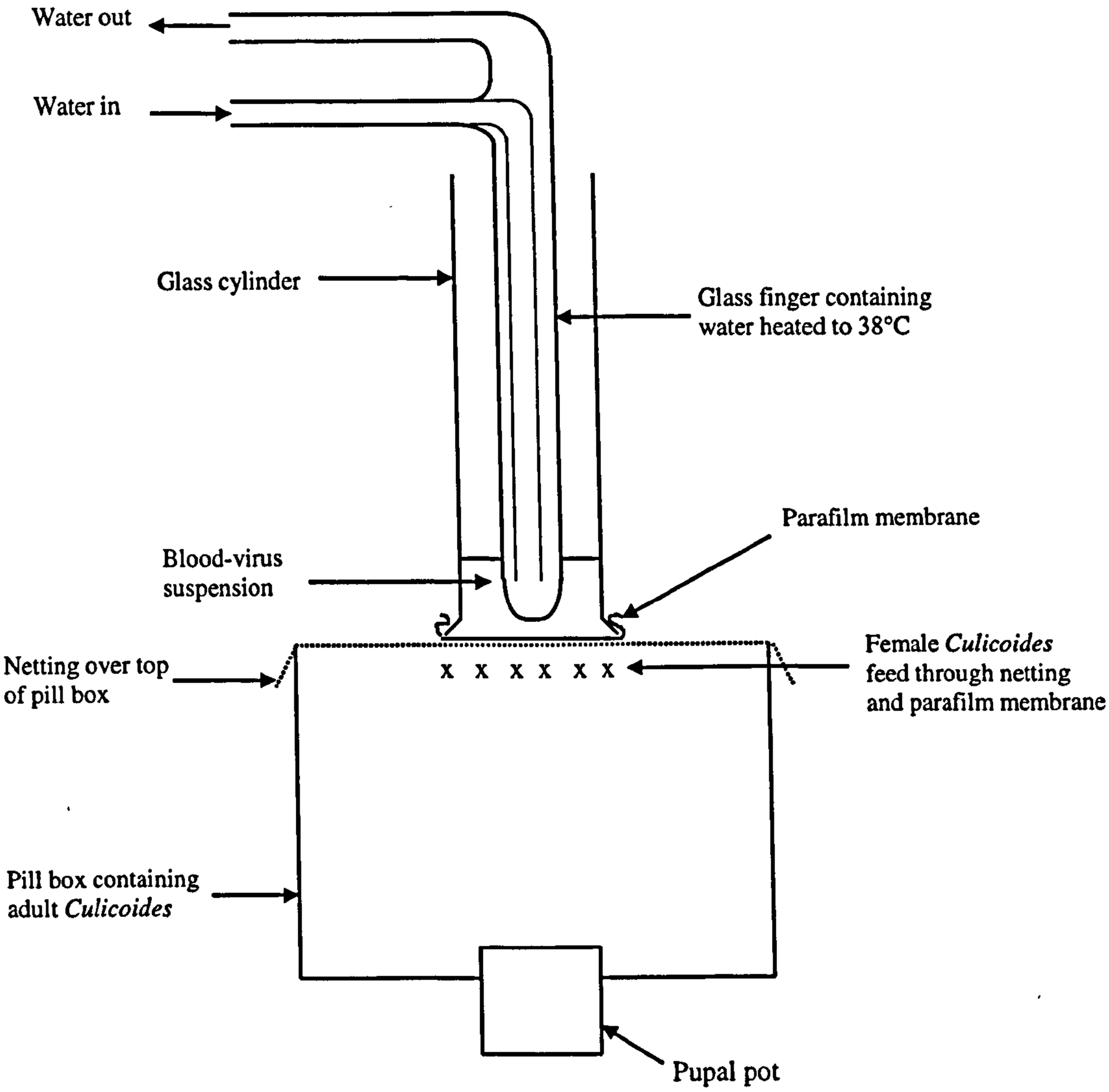


Figure 3.1 Blood-feeding apparatus. Feeding units consist of an outer glass cylinder and an inner glass 'finger'. A parafilm membrane is stretched across the bottom of the glass cylinder and the blood-virus suspension is added. Water, heated to 38°C, circulates through the glass finger and warms the blood-virus suspension. Female *Culicoides* blood-feed through the parafilm membrane.

effect of temperature on virus development) and 10 individual females (to determine the duration of the EIP and the proportion of females that were capable of viral transmission) were taken immediately after the blood-meal and then every 2 days for 28 days. After this time at 15°C, surviving females were transferred to 25°C for a further 10 days, with samples taken on days 29-31, 35 and 38. At 25 and 30°C, samples were taken immediately after the blood-meal and then daily for 10 days. In addition, at 25°C samples were taken on days 12 and 15. The midge samples were placed into 1.5 ml Eppendorf tubes and kept at -70°C until they could be assayed for virus.

Virus titration assay

Midge samples were ground up using motor-driven 1.5 ml polypropylene pestles (Anachem), in 0.5 ml of Glasgow Minimum Essential Medium (MEM; Life Technologies) which contained 200 µg/ml of penicillin/streptomycin and 2.5 µg/ml of fungizone. Two hundred microlitres of the midge suspensions was then diluted 1 in 10 in the MEM. Five additional ten-fold dilutions for the pools of midges and two additional ten-fold dilutions for the individual midges were then prepared.

Virus titrations were carried out in 96-well microtitre plates. Each well contained a monolayer of BHK-21 cells and 100 µl of MEM supplemented with 2% foetal calf serum, 100 µg/ml of penicillin/streptomycin and 2.5 µg/ml of fungizone. One hundred microlitres of each midge dilution was inoculated onto either each of six microtitre plate wells (pooled midges) or onto five wells (individual midges). The inoculated microtitre plates were subsequently incubated at 37°C in a carbon dioxide incubator. After five days the plates were observed microscopically for cytopathic effects which were used as a positive indicator of the presence of virus. Viral titres were then calculated using a method adapted from Spearman (1908) and Kärber (1931) and expressed as $TCID_{50}$/5 midges for pools of midges (Appendix 1.1) or $TCID_{50}$/midge for individuals (Appendix 1.2). The assay could detect viral titres between 1.367 and 7.2 $\log_{10}$ $TCID_{50}$/5 midges and between 1.4 and 4.2 $\log_{10}$ $TCID_{50}$/midge. Samples with viral titres <1.367 $\log_{10}$ $TCID_{50}$/5 midges or 1.4 $\log_{10}$ $TCID_{50}$/midge were classed as negative.

Extrinsic incubation period

Jennings and Mellor (1987) found that individual *C. variipennis sonorensis* containing >2.5 $\log_{10}$ $TCID_{50}$ of BTV4, after 8 days incubation at 25°C, regularly transmitted virus. Hence, the EIP was defined as the interval between the viraemic blood-meal and when the virus had replicated to a titre of >2.5 $\log_{10}$ $TCID_{50}$/midge. However, since some individuals imbibed >2.5 $\log_{10}$ $TCID_{50}$ of virus with the blood-meal, it was necessary to distinguish midges in which virus had replicated from those which had recently ingested virus. Typically, after ingestion of a viraemic blood-meal, the viral titre per midge decreases during an 'eclipse phase' but then rises as the virus replicates in the vector. Hence individuals with >2.5 $\log_{10}$ $TCID_{50}$ of virus were classed as potential transmitters once the eclipse phase had passed.

The time required for the first female to complete the EIP (i.e. minimum EIP) was determined at each temperature, for the different viruses. In addition, the median time required for females to complete the EIP was calculated at each temperature, for the different viruses. To do this, the time-cumulative proportion of females with viral titre >2.5 $\log_{10}$ $TCID_{50}$ was transformed using probit analysis (used to linearise cumulative curves) and plotted against the logarithm of days. Linear regression was then used to fit the best straight line and from this an estimate of the median EIP was obtained (Figure 3.2).

Extrinsic incubation rates (EIR; reciprocal of the EIP), when the first female and 50% of females were capable of virus transmission, were then calculated and regressed as linear functions of temperature. The theoretical minimum temperature for virus development (in the first female and 50% of females) was estimated by extrapolation of the regression lines to the x-axis and the number of day degrees above the minimum temperature required for virus development was estimated as the reciprocal of the slope of the lines (Figure 3.2). Finally, the regression lines for the first and median transmission rates were compared among the viruses by analysis of covariance (ANCOVA).

Figure 3.2 Procedure for calculating the median extrinsic incubation period and theoretical minimum temperature for virus development.

1. Calculation of time-cumulative proportion of midges with viral titre >2.5 $\log_{10}$ $TCID_{50}$ and probit transformation. In the example (which is based on the AHSV4 data at 25°C; page 68) midges from day 4 onwards (after the eclipse phase) were classed as potential transmitters and 10 individual midges were sampled on each test day.

Day	No. midges with viral titre >2.5 $\log_{10}$ $TCID_{50}$ / total midges tested	Time-cumulative proportion of midges with viral titre >2.5 $\log_{10}$ $TCID_{50}$	Probit transformation of time-cumulative proportion of midges with viral titre >2.5 $\log_{10}$ $TCID_{50}$
4	2/10	2/51 = 0.0392	3.2401
5	4/10	6/51 = 0.1176	3.8132
6	7/10	13/51 = 0.2549	4.3409
7	5/10	18/51 = 0.3529	4.6226
8	10/10	28/51 = 0.5490	5.1231
9	6/10	34/51 = 0.6667	5.4307
10	8/10	42/51 = 0.8235	5.9289
12	3/10	45/51 = 0.8824	6.1868
15	6/10	51/51 = 0.9902*	7.3339
Total	51/90	-	-

*To include extreme data points (i.e. 100% value for females with viral titre >2.5 $\log_{10}$ $TCID_{50}$) the proportion has been recalculated as 1-1/(2n), where n is the total number of females with viral titre >2.5 $\log_{10}$ $TCID_{50}$ (i.e. 51).

2. Probit of time-cumulative proportion of potential transmitters is regressed against the logarithm of days. An estimate of the median EIP is then obtained from the regression line. Steps 1 and 2 are repeated at each temperature for the different viruses.

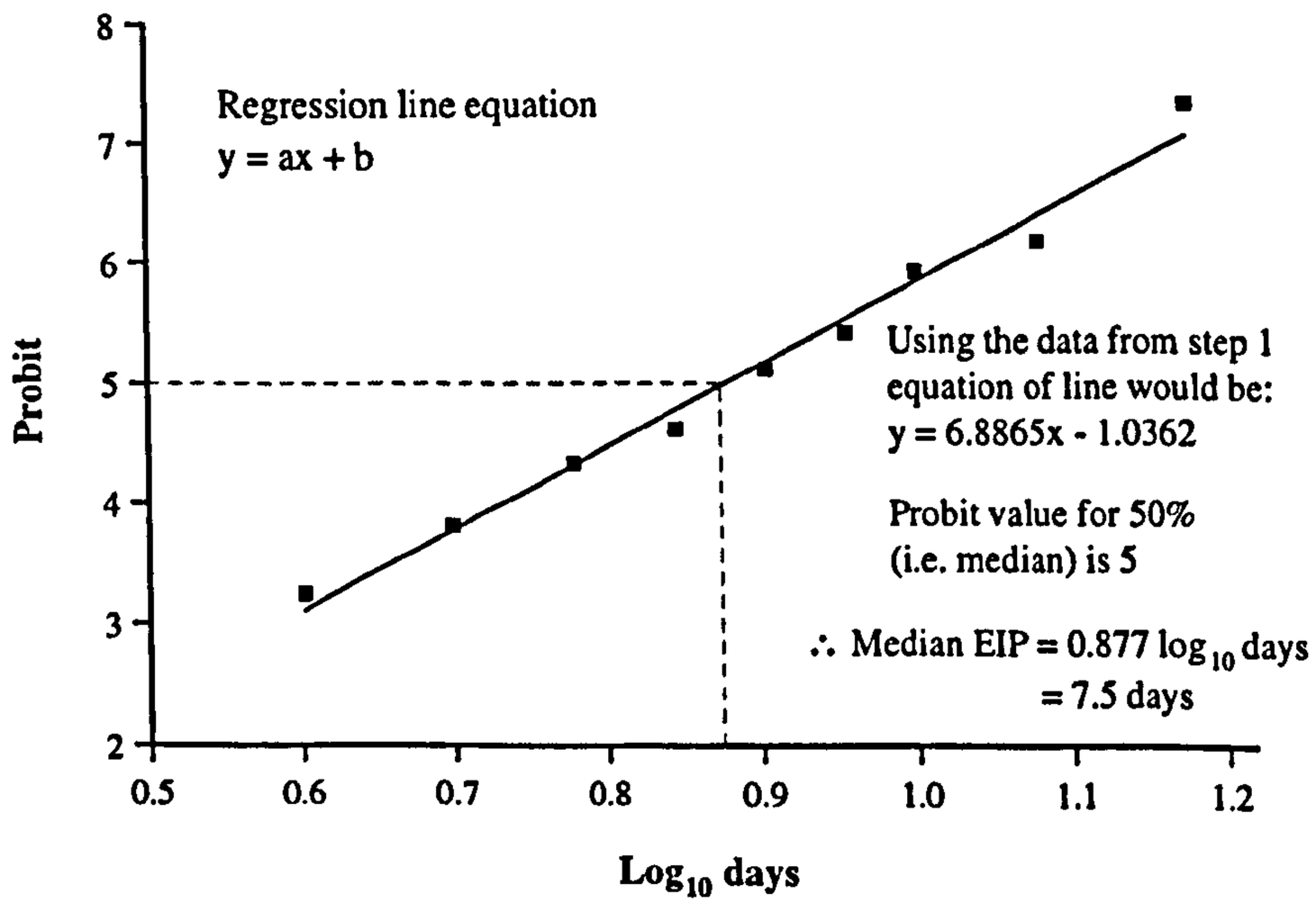


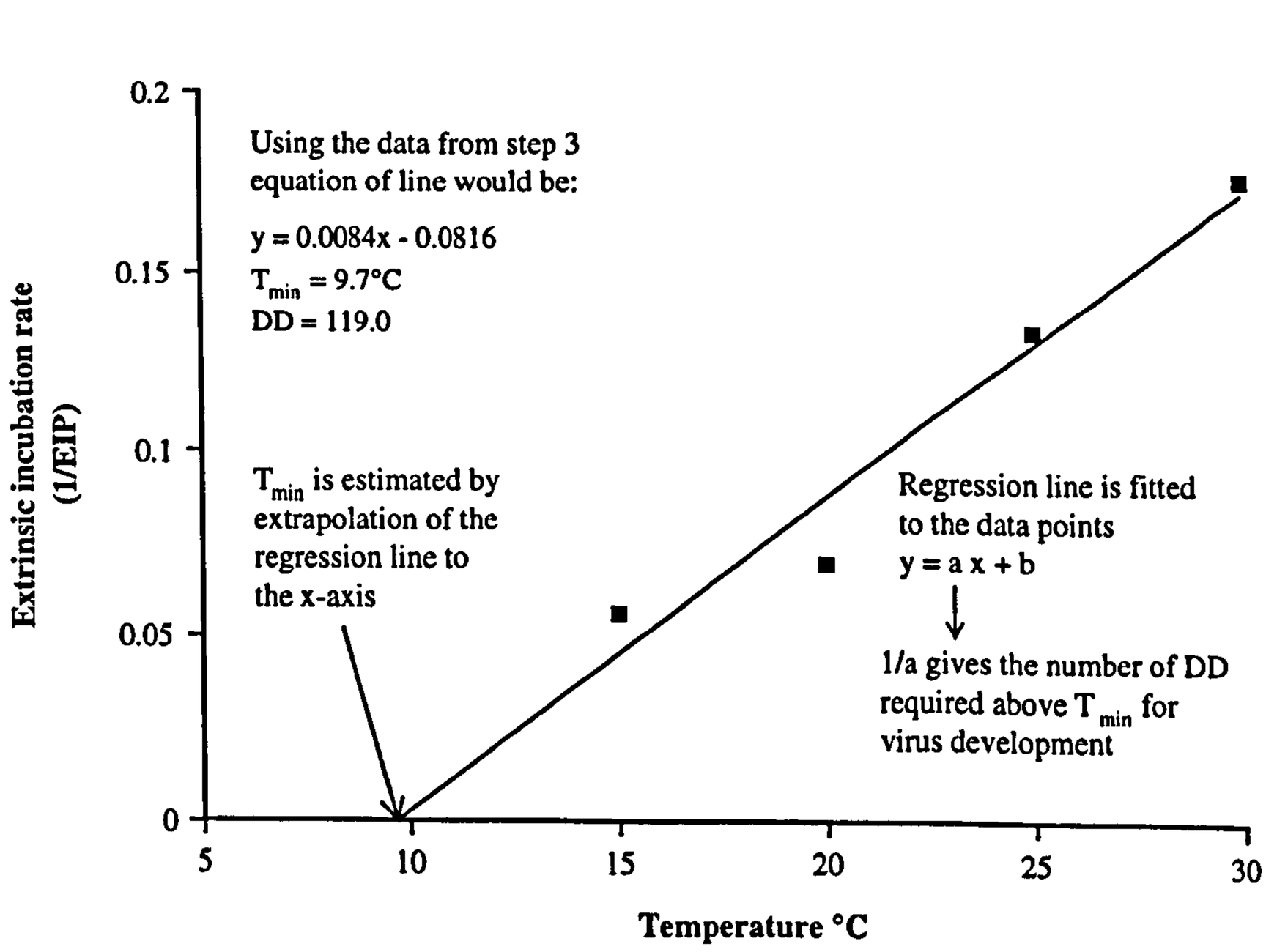
Figure 3.2 continued.

3. Calculation of extrinsic incubation rates (1/extrinsic incubation period)

Temperature °C	Estimated median EIP*	EIR
15	17.9	0.0559
20	14.4	0.0694
25	7.5	0.1333
30	5.7	0.1754

* or minimum EIP

4. Extrinsic incubation rates are regressed against temperature. The theoretical minimum temperature for virus development (T_{min}) and the number of day degrees (DD) required above T_{min} for virus development are estimated from the regression line. The duration of the EIP at constant or varying temperatures between 15 and 30°C can then be estimated using the equation EIP = DD / T – T_{min}, where T is the ambient temperature.



Vector competence

To establish the impact of temperature on the vector competence of *C. variipennis sonorensis* for the different viruses, the proportion of individual midges with a viral titre >2.5 $\log_{10}$ $TCID_{50}$ on each test day after the eclipse phase was transformed using the logit transformation and regressed against temperature. Binomial errors were used in the regression analysis to account for the use of proportion data (Crawley, 1993). Since there is no exact theory for the distribution of deviances in an analysis with non-normal error variances, χ^2 approximations were used. However, in a number of cases, data were overdispersed. A heterogeneity factor HF (HF = Pearson χ^2/df; Crawley, 1993) corrects for overdispersion and F-tests rather than χ^2 tests must then be used. The regression lines for the different viruses were compared by ANCOVA.

In addition, a one-way analysis of variance (ANOVA) with binomial errors was used to compare the proportion of females that could transmit virus at 25°C after initially being held at 15°C with the proportion of competent females that had been held entirely at 15 or 25°C.

Survival of adult *C. variipennis sonorensis*

One to two day old adult *C. variipennis sonorensis* were allowed to feed on a blood-virus suspension consisting of heparinised horse blood mixed with AHSV4. Groups of 100 blood-fed females were then placed into pill boxes and subsequently kept in exsiccators at 40, 75 or 85% relative humidity, at 15, 20, 25 or 30°C. Forty percent humidity was achieved using saturated solutions of $CaCl_2.6H_2O$ at 15°C and NaI at 20-30°C, while NaCl and KCl maintained the humidity at 75 and 85% respectively, across the temperature range (Winston and Bates, 1960). Midges were provided with a pad of cotton wool soaked in 10% sucrose solution medicated with antibiotics for one hour a day. This enabled the midges to feed but did not alter the humidity too greatly. The number of midges that died each day was then counted until all the midges had died.

Survival analysis with exponential errors (to describe a Type II survivorship curve where the risk of death is independent of age) and a reciprocal link was carried out on the times to death, to determine both the mean survival times and the daily survival

rates at each temperature/humidity combination. The effect of temperature and relative humidity on survival was then assessed using ANCOVA. The saturation deficit (Appendix 2), which provides a measure of the drying power of air based on both relative humidity and temperature, was also calculated for each temperature/humidity combination. Times to death were then regressed against temperature and saturation deficit.

3.4 Results

Virus development

The geometric mean AHSV titres in pools of *C. variipennis sonorensis* incubated at different temperatures are shown in Figure 3.3. In general, at each temperature, the mean AHSV4 titre declined after the blood-meal (eclipse phase) but then subsequently increased over time. This pattern is typical of arbovirus development in a vector and is related to the initial digestion and/or excretion of virus particles as well as the uncoating of virus particles during infection of the midgut cells (core particles are less infectious for BHK-21 cells than intact virus particles; Mertens *et al.*, 1996), followed by replication of the virus in the midgut cells and secondary target organs. However temperature did affect both the rate of AHSV4 development and the extent of viral replication. For example, at 25°C replicating virus was detected by day 4 and viral titres of ≈5 $\log_{10}$ $TCID_{50}$/5 midges were subsequently observed, whereas at 15°C, replication of AHSV4 was not detected until day 10 and viral titres of only ≈3-4 $\log_{10}$ $TCID_{50}$/5 midges were subsequently obtained.

AHSV6 development was limited regardless of the incubation temperature (Figure 3.3). At 15°C, AHSV6 was not detected between days 10 and 35, while at 25°C the maximum titre obtained after the eclipse phase was only 1.6 $\log_{10}$ $TCID_{50}$/5 midges which is not even sufficient for an individual midge to be capable of viral transmission. Thus since the PIRB-s-3 strain of *C. variipennis sonorensis* appeared to be refractory to this strain of AHSV6, the trials at 20 and 30°C were not completed.

BTV10 and BTV16 developed more rapidly at the higher temperatures, although the results at 25°C are difficult to interpret due to considerable variation in the viral titres (Figure 3.4). EHDV1 development was also affected by temperature and at 15°C the

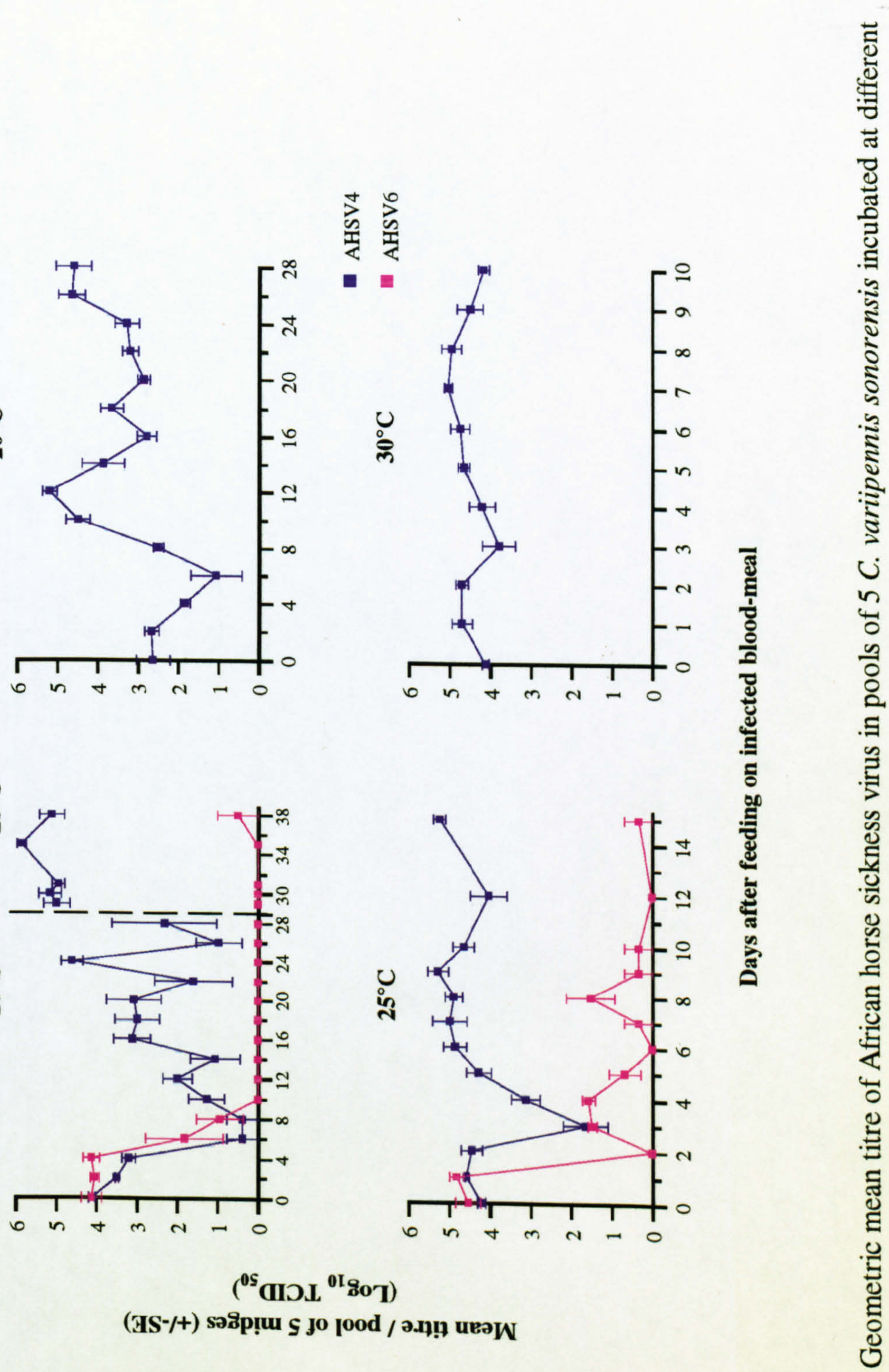


Figure 3.3 Geometric mean titre of African horse sickness virus in pools of 5 *C. variipennis sonorensis* incubated at different temperatures (based on 4 pools of 5 midges per test day).

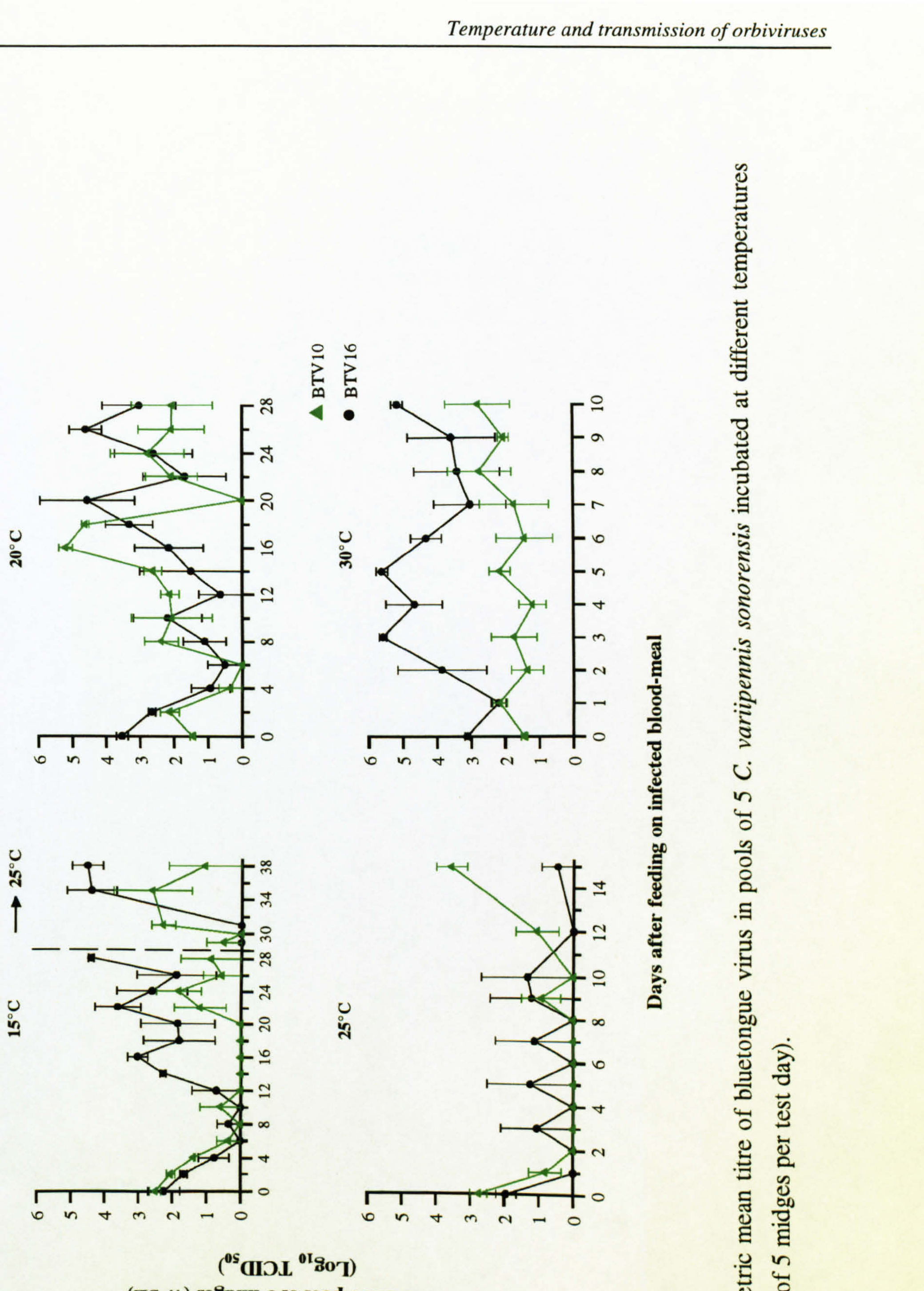


Figure 3.4 Geometric mean titre of bluetongue virus in pools of 5 *C. variipennis sonorensis* incubated at different temperatures
(based on 4 pools of 5 midges per test day).

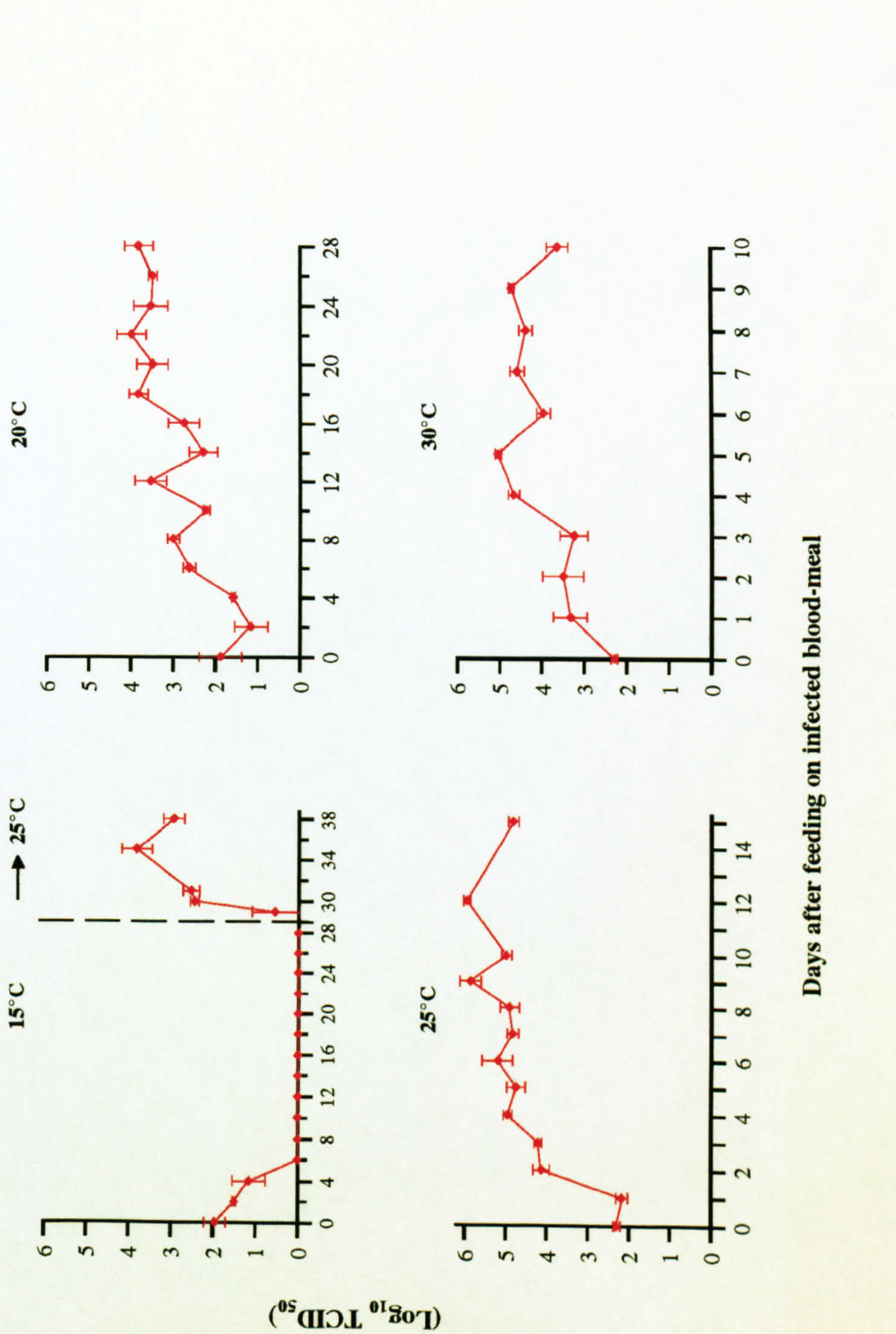


Figure 3.5 Geometric mean titre of epizootic haemorrhagic disease virus serotype 1 in pools of 5 *C. variipennis sonorensis* incubated at different temperatures (based on 4 pools of 5 midges per test day).

Table 3.2 Estimated duration of the EIP for different orbivirus serotypes in *C. variipennis sonorensis* maintained at different temperatures. Minimum and median EIPs refer to the time interval between ingestion of the viraemic blood-meal and when the first and 50% of competent females respectively were capable of viral transmission.

Temperature °C	EIP - Days							
	AHSV4		BTV10		BTV16		EHDV1	
	Minimum	Median	Minimum	Median	Minimum	Median	Minimum	Median
15	14	17.9	26	26	16	19.9	-	-
20	6	14.4	8	13.0	10	20.2	6	18.2
25	4	7.5	15	15	4	7.2	2	5.7
30	4	5.7	3	7.0	2	4.8	2	4.8

virus was unable to replicate until midges were subsequently transferred to 25°C (Figure 3.5).

Extrinsic incubation period

The proportion of individual *C. variipennis sonorensis* with a viral titre >2.5 $\log_{10}$ $TCID_{50}$/midge at the different temperatures is shown in Figure 3.6. Using these data, the minimum and median EIPs for the different viruses at each temperature were estimated (Table 3.2). The minimum and median EIPs for BTV10 at 15°C were the same, as only one individual at this temperature was potentially capable of transmitting the virus. This was also the case at 25°C. In addition, the minimum and median EIPs for AHSV6 at 15 and 25°C could not be estimated, as none of the midges developed a viral titre >2.5 $\log_{10}$ $TCID_{50}$/midge.

The rate of development (i.e. EIR) of AHSV4, BTV10, BTV16 and EHDV1 in *C. variipennis sonorensis* increased linearly between 15 and 30°C (Figure 3.7). The theoretical minimum temperature for virus development in the first female varied from 7.6°C for AHSV4 to 14.7°C for BTV16, while in 50% of females it varied from 9.1°C for BTV10 to 15.1°C for EHDV1. The number of day degrees required for

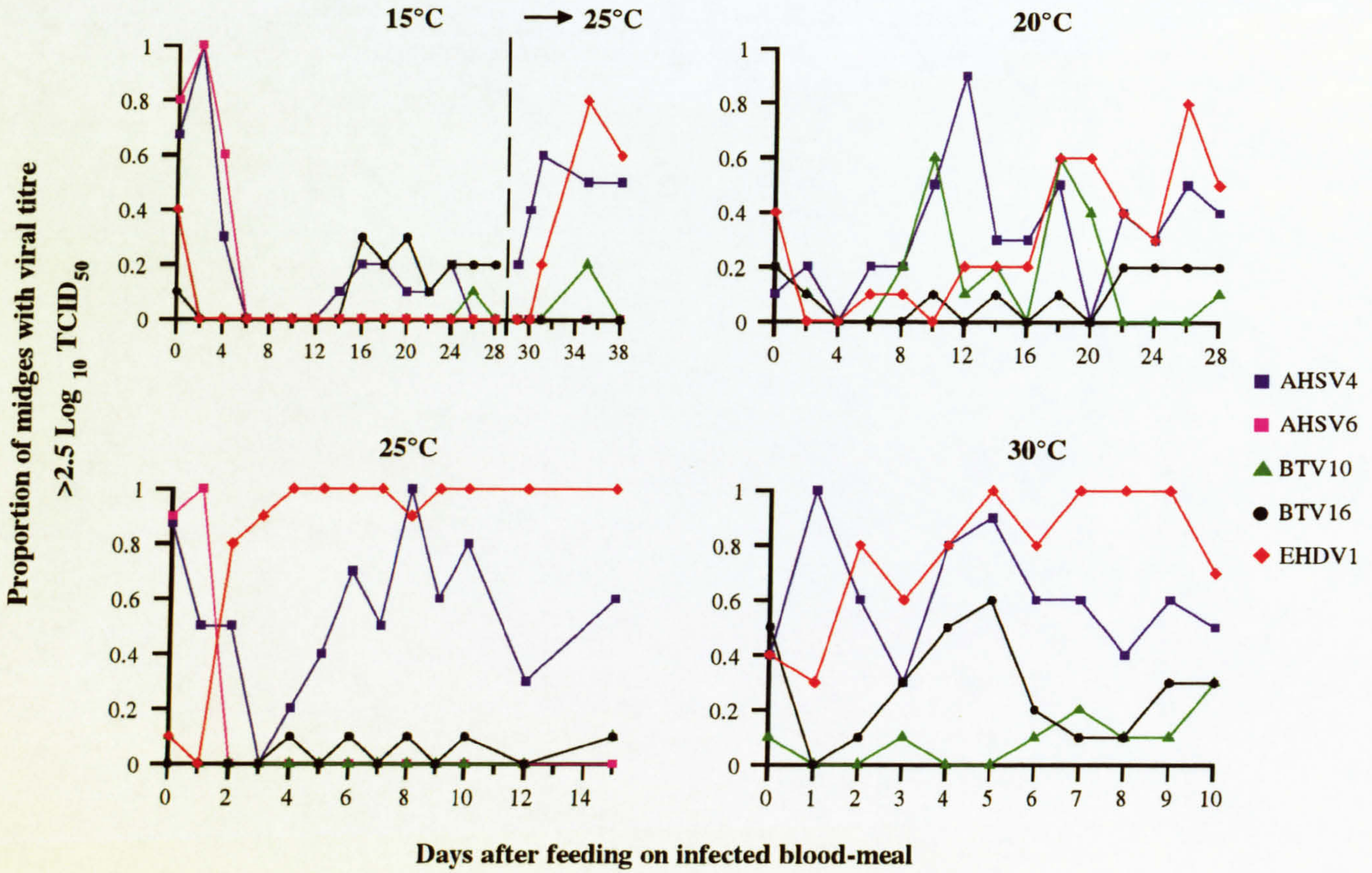


Figure 3.6 Proportion of individual *C. variipennis sonorensis* with a viral titre >2.5 $\log_{10}$ $TCID_{50}$ at different temperatures. Ten midges were sampled on each test day and those individuals with a viral titre >2.5 $\log_{10}$ $TCID_{50}$ after the eclipse phase of virus development were considered to be capable of viral transmission e.g. for AHSV4 individuals from days 14, 6, 4 and 4 onwards at 15, 20, 25 and 30°C respectively were classed as potential transmitters. Median EIPs were calculated from the data according to Figure 3.2.

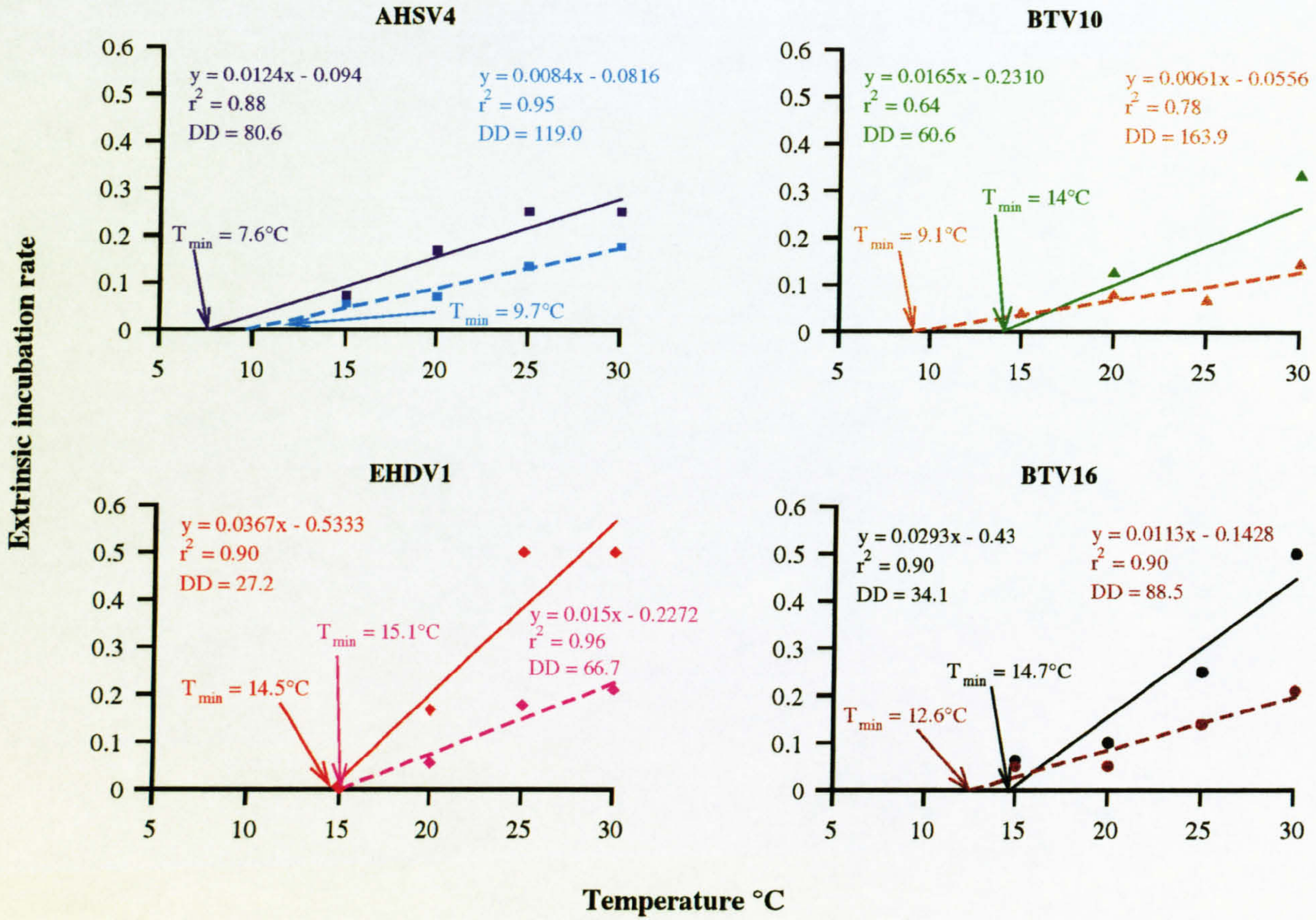


Figure 3.7 Extrinsic incubation rates (1/extrinsic incubation period) when the first (solid lines) and 50% (dashed lines) of female *C. variipennis sonorensis* could transmit orbiviruses, at different temperatures. T_{min} is the theoretical minimum temperature for virus development and DD is the total number of day degrees required above T_{min} for virus development.

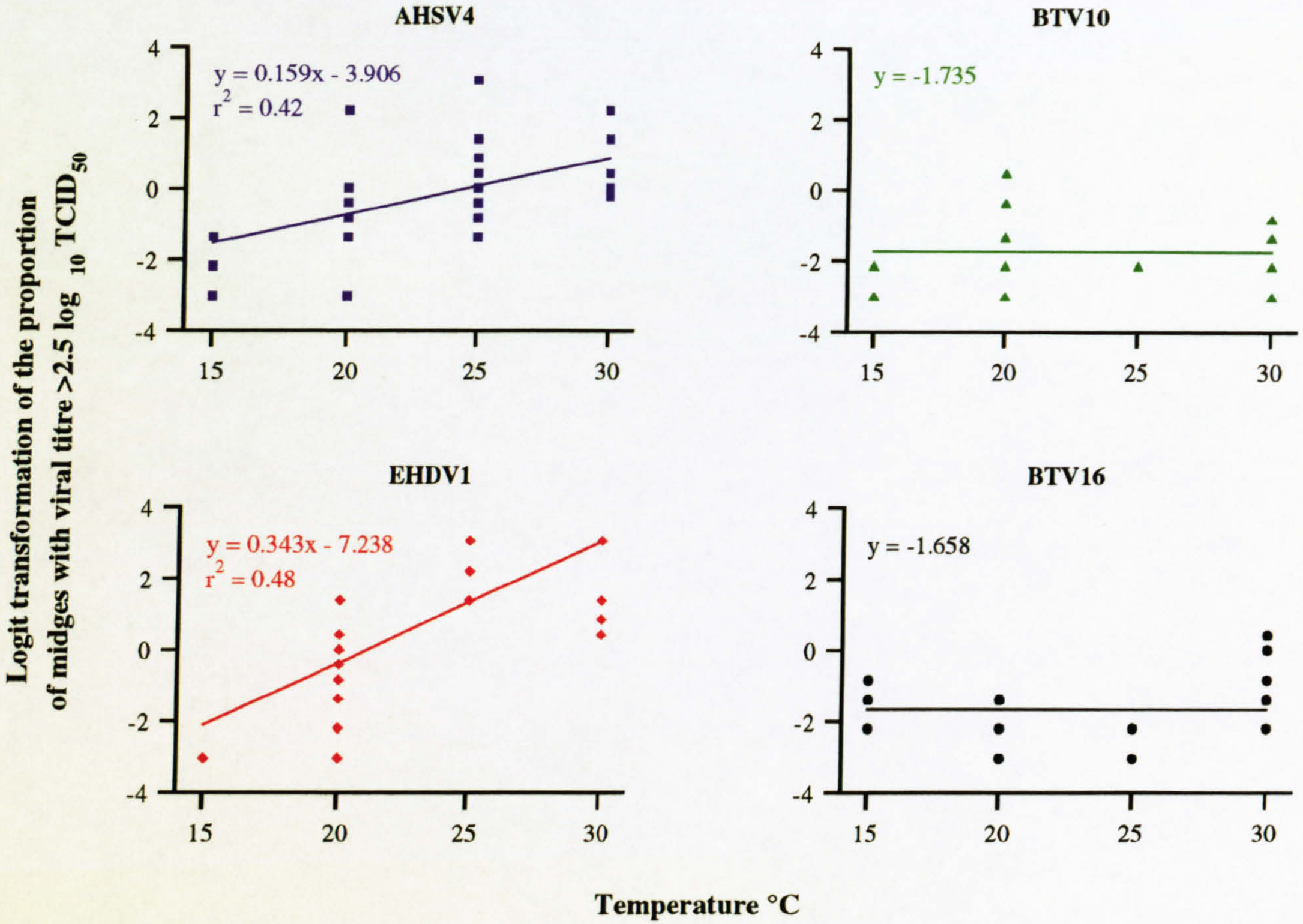


Figure 3.8 Effect of temperature on the vector competence of *C. variipennis sonorensis* for different orbiviruses. To describe the relationship between temperature and vector competence, the proportion of individual midges with a viral titre >2.5 $\log_{10}$ $TCID_{50}$ from each test day after the eclipse phase (see Figure 3.6) was transformed using the logit transformation and regressed against temperature.

development of AHSV4 and BTV10 in *C. variipennis sonorensis* was substantially greater than that required for the development of BTV16 or EHDV1 (Figure 3.7). The regression lines describing the relationship between temperature and the EIR for virus development in the first female ($F_{3,8} = 2.41$, NS) or 50% of females ($F_{3,8} = 2.99$, NS) did not differ significantly among the serotypes.

Vector competence

The vector competence of *C. variipennis sonorensis* for AHSV4 ($F_{1,34} = 15.82$, $p < 0.01$) and EHDV1 ($F_{1,29} = 6.50$, $p < 0.05$) increased linearly between 15 and 30°C (Figure 3.8). However, vector competence for BTV10 ($F_{1,20} = 0.01$, NS) and BTV16 ($F_{1,33} = 0.69$, NS) was not significantly affected by these temperatures (Figure 3.8). The effect of temperature on vector competence differed significantly among the virus species ($F_{1,119} = 3.93$, $p < 0.05$).

When *C. variipennis sonorensis* were transferred to 25°C after initially being held at 15°C (Figure 3.6), the proportion of midges that were capable of transmitting AHSV4 increased significantly ($\chi^2 = 40.47$, df = 1, $p < 0.001$), to a level similar to that for midges which had been held continuously at 25°C. Although transfer to 25°C increased the susceptibility of midges to EHDV1, the proportion of midges able to transmit EHDV1 was still greater when they were held continuously at 25°C ($\chi^2 = 671.6$, df = 2, $p < 0.001$). The proportion of midges able to transmit BTV10 was not significantly affected by these temperatures ($\chi^2 = 0.53$, df = 2, NS), while for BTV16 the proportion of competent midges was lower at 15/25°C compared to 15 and 25°C ($\chi^2 = 14.26$, df = 1, $p < 0.001$).

Survival of adult C. variipennis sonorensis

Survival of adult *C. variipennis sonorensis* (Table 3.3) decreased significantly as temperature increased from 15 to 30°C ($\chi^2 = 201.7$, df = 1, $p < 0.001$), but was not significantly affected by saturation deficit ($\chi^2 = 0.97$, df = 1, NS) or relative humidity ($\chi^2 = 0.03$, df = 2, NS). However, the interaction between relative humidity and temperature was significant ($\chi^2 = 15.6$, df = 1, $p < 0.001$), so that at low temperatures survival was greater at high relative humidity (85% RH) compared to lower relative humidities (40 and 75% RH), but at high temperatures the impact of relative humidity

Table 3.3 Survival of blood–fed female *C. variipennis sonorensis* at different temperatures and relative humidities. Females were 1-2 days old at the time of the blood-meal, which consisted of horse blood mixed with AHSV4. One hundred females were kept at each temperature/humidity combination.

Temperature °C	% Relative humidity	Saturation deficit mbar	Survival range days	Mean survival days	+SE	-SE	Survival rate / day*
15	40	10.3	2 – 52	27.3	3.1	2.5	0.96
	75	4.3	1 – 46	27.5	1.4	1.3	0.96
	85	2.6	4 – 57	33.2	1.4	1.3	0.97
20	40	14.1	2 – 33	15.6	1.8	1.5	0.94
	75	5.9	2 – 31	18.8	0.8	0.7	0.95
	85	3.5	1 – 41	20.5	0.8	0.8	0.95
25	40	19.1	2 – 26	14.4	1.5	1.3	0.93
	75	8.0	2 – 23	13.4	0.6	0.6	0.93
	85	4.8	2 – 18	10.9	0.6	0.5	0.91
30	40	25.7	2 – 20	11.9	1.3	1.1	0.92
	75	10.7	2 – 15	10.2	0.5	0.5	0.91
	85	6.4	2 – 12	7.1	0.4	0.4	0.87

*daily survival rate is $e^{-(1/\text{mean survival})}$

was reversed (Table 3.3). The following regression equations describe the effect of temperature and relative humidity on survival at temperatures between 15 and 30°C:

Low humidity (40 and 75% RH):

$y = 0.0036x - 0.0165$, $r^2 = 0.38$, $\chi^2 = 86.6$, df = 1, $p < 0.001$

High humidity (85% RH):

$y = 0.0064x - 0.0681$, $r^2 = 0.60$, $\chi^2 = 130.7$, df = 1, $p < 0.001$

where x is temperature and y is the hazard function (i.e. instantaneous death rate).

Virus transmission

Using equation 1 (page 54), the proportion of female *C. variipennis sonorensis* that could take at least one virus transmissive blood-meal for the different orbivirus

serotypes at 15-30°C was calculated. The equations derived for the impact of temperature on 1. duration of the minimum and median EIP (Figure 3.7; EIR = 1/EIP) 2. vector competence (Figure 3.8; logit = ln(p/1-p) where p is the proportion of competent midges; Crawley, 1993) and 3. survival rates at low (40 and 75% RH) and high humidities (85% RH) (page 72; hazard function is -ln(daily survival rate)) were used for the calculations. The blood-feeding interval (to determine the number of days to the first blood-meal after the minimum or median EIP) was calculated using the equation $y = -1.98 + 0.07217x + 2516.65/x^2$, where x is temperature and y is the mean number of days to oviposition (Mullens and Holbrook, 1991), since *Culicoides* are capable of taking their next blood-meal on the same day as oviposition. The lowest temperature at which females could take a virus transmissive blood-meal was considered to be the minimum temperature for virus transmission, while the optimum temperature for virus transmission was considered to be the temperature at which the greatest proportion of females could take a virus transmissive blood-meal.

The proportion of female *C. variipennis sonorensis* that could take at least one virus transmissive blood-meal increased between 15 and 30°C for AHSV4, EHDV1, BTV10 and BTV16, although the magnitude of the increase was dependent on the virus (Figure 3.9). Estimates of the minimum and optimum temperatures for transmission of these viruses by *C. variipennis sonorensis* are shown in Table 3.4.

The lowest temperature at which female *C. variipennis sonorensis* could take a virus transmissive blood-meal was largely determined by the relationship between adult survival and the duration of the EIP. For example, while EHDV1 development in 50% of females only ceases at 15.1°C, transmission below 17°C is unlikely because few females can survive the lengthy EIP (>36 days) at temperatures <17°C. However, for some of the viruses (e.g. AHSV4 and BTV10 and BTV16 for calculations involving the median EIP) the minimum temperature for virus transmission could only be estimated to occur within a range of temperatures (Table 3.4). Thus although the theoretical minimum temperature for development of these viruses was estimated to be <15°C, adult survival could only be calculated at 15-30°C. The optimum temperature for virus transmission varied between 27 and 30°C depending on the virus (Table 3.4), although if trials had been carried out at >30°C, it is possible that the optimum could be even higher. Trials at higher temperatures are also necessary to

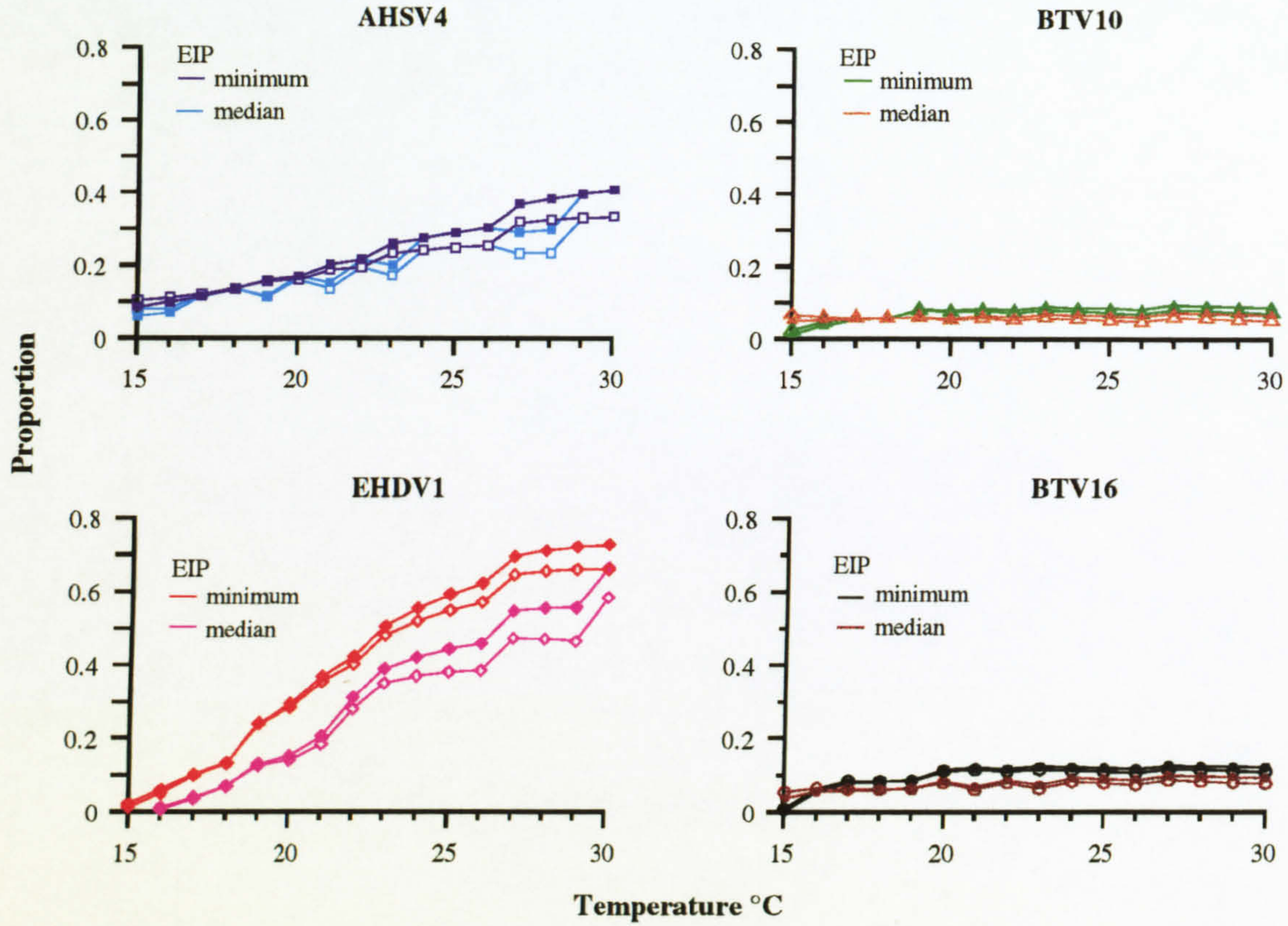


Figure 3.9 Effect of temperature on the proportion of female *C. variipennis sonorensis* that could take at least one virus transmissive blood-meal, for different orbivirus serotypes. The proportion of females that could transmit virus was calculated using equation 1. The minimum and median EIPs and survival rates at low (solid symbols) and high (open symbols) humidities, as well as mean values for vector competence and the blood-feeding interval were used. The temperatures at which the greatest proportion of females could take at least one virus transmissive blood-meal were considered to be the optimum for virus transmission (e.g. 30°C for EHDV1 for calculations using the median EIP; pink lines). The lowest temperature at which females were able to take a virus transmissive blood-meal was considered to be the minimum temperature for virus transmission (e.g. 17°C for EHDV1 for calculations using the median EIP; pink lines).

Table 3.4 Minimum and optimum temperatures for transmission of different orbivirus species and serotypes by *C. variipennis sonorensis*.

Serotype	EIP	Minimum °C	Optimum °C
AHSV4	min median	7.6-15 9.7-15	28-30+ 29-30+
BTV10	min median	15 9.1-15	27-30+ 27-30+
BTV16	min median	16 12.6-15	27-30+ 27-30+
EHDV1	min median	15 17	28-30+ ≥30

determine the upper temperature limit for virus transmission, although this is likely to be dependent on vector survival, as virus development is rapid at high temperatures.

3.5 Discussion

The impact of temperature on the duration of the EIP, vector competence and vector survival greatly affected the transmission potential of *C. variipennis sonorensis*, for those orbivirus species and serotypes to which it was genetically susceptible (i.e. AHSV4, EHDV1, BTV10 and BTV16). High temperatures favoured transmission of these viruses, with a greater proportion of midges capable of taking at least one virus transmissive blood-meal at 27-30°C, than at temperatures ≤15°C. However, the extent to which temperature could affect the transmission potential of *C. variipennis sonorensis* was dependent on the orbivirus species and serotype.

Temperature and the EIP: The duration of the EIP for AHSV4, EHDV1, BTV10 and BTV16 in *C. variipennis sonorensis* was shorter at higher temperatures. Consequently, female *C. variipennis sonorensis* would be able to transmit virus sooner when conditions are warm. However, despite the consistent affect of temperature on the rate of virus development, the time required for development did vary among the virus species and serotypes. For example, the number of day degrees

required for development of AHSV4 and BTV10 in *C. variipennis sonorensis* was substantially greater than that required for either BTV16 or EHDV1.

Although virus development was limited at low temperatures, virus titres in pools of midges increased when *C. variipennis sonorensis* were transferred to 25°C, after previously being incubated for 28 days at 15°C. In addition, a greater proportion of individual midges were capable of transmitting AHSV4 and EHDV1 after transfer to 25°C. Thus it appears that virus can persist in *C. variipennis sonorensis* for extensive periods at low temperatures and that subsequent exposure to warm temperatures can result in some midges developing full virus infections. This phenomenon has also been observed in *C. variipennis sonorensis* infected with BTV11 (Mullens *et al.*, 1995) and AHSV9 (Wellby *et al.*, 1996), while in other arbovirus/vector systems, it has been shown that virus remains in the midgut cells of vectors incubated at low temperatures, but then spreads to secondary target organs following exposure to higher temperatures (e.g. Shichijo *et al.*, 1972).

Temperature and vector competence: The effect of temperature on the vector competence of *C. variipennis sonorensis* (i.e. the proportion of midges with competent genotypes that develop competent phenotypes) varied greatly among the viruses. Thus while temperature did not affect the vector competence of midges for BTV10 and BTV16, the ability of *C. variipennis sonorensis* to transmit both AHSV4 and EHDV1 increased between 15 and 30°C, although EHDV1 showed a greater response. Earlier studies also indicate that the effect of temperature on vector competence can be variable, for example, the infection rate of *C. variipennis sonorensis* with BTV11 was similar at temperatures between 21 and 32°C (Mullens *et al.*, 1995), while the infection rate with AHSV9 increased with temperature (Wellby *et al.*, 1996).

The PIRB-s-3 strain of *C. variipennis sonorensis* appeared to be refractory to AHSV6. Since virus was not detected (even at low level) in the majority of individuals sampled, it is likely that these *C. variipennis sonorensis* possessed a midgut infection barrier to AHSV6. In addition, when AHSV6 was inoculated directly into the haemocoel, 100% of midges developed viral titres of >2.5 $\log_{10}$ $TCID_{50}$/midge (data

not shown), further indicating that the barrier to infection was associated with the midgut.

Temperature and survival: The daily survival rate of adult *C. variipennis sonorensis* decreased with increasing temperature and on average midges lived three times longer at 15°C than at 30°C. Mellor *et al.* (2000) speculated that reduced survival at higher temperatures may be due to desiccation rather than temperature *per se*, since saturation deficit increases with temperature. However in this study, higher mortality at elevated temperatures appeared to be attributable to temperature, as saturation deficit (ranging from 2.6 to 25.7 mbar) did not directly influence adult survival.

However, relative humidity did affect adult survival at the different temperatures, such that at low temperatures low humidity was detrimental for survival, while at high temperatures high humidity was detrimental. At low temperatures, the reduction in longevity of *C. variipennis sonorensis* at low humidities may have been due to excessive water loss. In addition, since the drying power of air increases with temperature, it was expected that the detrimental effect of low humidity would be even greater at high temperatures. Indeed, Murray (1991) demonstrated that the detrimental impact of low humidity on *C. brevitarsis* survival increased with temperature. The finding that low humidity actually increased survival at high temperatures was therefore surprising. However, it is possible that at the higher humidities, conditions were too moist, hindering the ability of midges to eliminate excess metabolic water by evaporation. There was no evidence to suggest that fungi or other pathogens (which thrive in warm moist conditions) were the cause of the increased mortality.

Temperature and transmission: The transmission potential of *C. variipennis sonorensis* for the different viruses was largely dependent on the relationship between adult survival and the time to the first virus transmissive blood-meal. Hence high temperatures (e.g. 27-30°C) favoured transmission of AHSV4, EHDV1, BTV10 and BTV16, because although adult longevity was reduced the duration of the EIP was sufficiently fast that a greater proportion of midges could survive to transmit virus. In addition, the impact of high temperatures on transmission of AHSV4 and EHDV1 was enhanced by the increased vector competence of *C. variipennis sonorensis* for these

serotypes. In contrast, at cooler temperatures (e.g. 15-17°C), adult longevity was extended but the EIP was disproportionately prolonged, so that few midges could survive the incubation period, while the vector competence of *C. variipennis sonorensis* for all the viruses was low. At temperatures below the theoretical minimum for virus development (e.g. 7.6-15.1°C depending on the virus) viral transmission even at low level is impossible. However, if temperatures subsequently increase, latent virus can replicate, increasing the potential for transmission. Indeed, Wellby *et al.* (1996) suggested that the persistence of virus in the vector for long periods at low temperatures and the subsequent resumption of viral development on exposure to warm conditions may provide an overwintering mechanism for these viruses, since vector longevity is also extended at low temperatures (e.g. up to 90 days in some cases; Mellor *et al.*, 2000).

While the findings of this study indicate that temperature and humidity can greatly affect the transmission of orbiviruses by *C. variipennis sonorensis*, it must be borne in mind that midges were maintained at constant temperatures and relative humidities. In the field midges will experience a range of temperatures and instead of being restricted to a particular humidity which may be detrimental for survival, they can move to habitats with more suitable microclimates. In addition, the impact of temperature and humidity on the transmission potential of the PIRB-s-3 strain of *C. variipennis sonorensis* for different orbiviruses may not be representative of the impact on other *C. variipennis sonorensis* populations, since different populations are likely to vary in their susceptibility to viruses (e.g. Jones and Foster, 1978) and in their response to temperature. For these reasons, the specific effects of temperature and humidity on the transmission potential of other *Culicoides* species may also be difficult to predict. However, experiments such as this provide a useful insight into the impact that temperature and humidity can have on transmission of orbiviruses by *Culicoides* and can be used to broadly assess the risk of orbivirus transmission occurring within a particular region.

CHAPTER 4: EFFECT OF TEMPERATURE ON THE TRANSMISSION OF AFRICAN HORSE SICKNESS VIRUS SEROTYPE 8 BY *CULICOIDES IMICOLA* (DIPTERA: CERATOPOGONIDAE).

4.1 ABSTRACT

The effect of temperature on the transmission of African horse sickness virus serotype 8 (AHSV8) by *Culicoides imicola* was investigated. The vector competence of *C. imicola* for AHSV8 did not vary significantly at incubation temperatures of 15, 18, 26 or 30°C and overall only 2% of *C. imicola* were considered capable of transmitting virus. However, the development time of AHSV8 in *C. imicola* was affected by temperature and the duration of the extrinsic incubation period was shorter at higher temperatures. The minimum temperature for AHSV8 development in *C. imicola* was estimated to be 9.9°C. These findings can be used to identify regions of Europe that have suitable conditions for AHSV transmission, both currently and if conditions should warm with climate change.

4.2 INTRODUCTION

African horse sickness is a non-contagious, infectious, arthropod-borne disease of equids and in susceptible horse populations mortality rates may be as high as 95% (Coetzer and Erasmus, 1994). The disease is caused by African horse sickness virus (AHSV), a dsRNA virus (Reoviridae: Orbivirus), of which there are nine internationally recognised serotypes. AHSV is enzootic in parts of sub-Saharan Africa but sporadic outbreaks have also occurred in southern Europe, for example in Spain during 1966 (Diaz Montilla and Panos Marti, 1968) and in Spain and Portugal between 1987 and 1990 (Rodriguez *et al.*, 1992).

The only confirmed field vector of AHSV is *Culicoides imicola* (Diptera: Ceratopogonidae). This species is principally Afro-Asian, but in 1982 it was recorded for the first time in Europe, from Cordoba in southern Spain (Mellor *et al.*, 1983). It is now known to occur across most of south-western Iberia, up to 41°17'N in Portugal and 40°N in Spain (Rawlings *et al.*, 1997). It has also been recorded from the Greek islands of Lesbos (Boorman and Wilkinson, 1983), Rhodes (Boorman, 1986), Chios, Kos, Samos (Mellor, pers. comm.) and Evia (Patakakkis, unpub. obs.) and was

recently discovered on mainland Greece, in the provinces of Chaldithiki, Larisa and Magnisia (Patakakkis, unpub. obs.).

The ability of *Culicoides* populations to transmit AHSV is affected by environmental factors, such as temperature (Chapter 3). Consequently, to identify the regions of Europe where AHSV transmission could occur, both currently and if conditions should warm (as predicted by climate change scenarios; Intergovernmental Panel on Climate Change, 1996), it is essential to quantify the effect of temperature on AHSV transmission by *C. imicola*. However, *C. imicola* has proved impossible to rear in the laboratory and earlier work into the effect of temperature on AHSV transmission has therefore focused on *C. variipennis sonorensis*, which has been colonised (Boorman, 1974) and is also susceptible to AHSV (Boorman *et al.*, 1975; Mellor *et al.*, 1975).

These studies showed that AHSV was unable to replicate in *C. variipennis sonorensis* at temperatures below ≈10°C, while within favourable temperature limits, the duration of the extrinsic incubation period (EIP; the development time of the virus in the vector) was inversely related to temperature (Wellby *et al.*, 1996; Chapter 3). Furthermore, a greater proportion of midges could survive to transmit virus at higher temperatures (e.g. 28-30°C), because although longevity of *C. variipennis sonorensis* was reduced at high temperatures, this was more than compensated for by the shorter EIP (Chapter 3). In addition, the vector competence of this *C. variipennis sonorensis* population for AHSV4 (i.e. proportion of midges capable of transmitting virus) increased linearly with temperature (Chapter 3).

While these studies have provided useful insights into the influence of temperature on AHSV transmission by *Culicoides*, it is essential to confirm these findings in *C. imicola*. Recently a method was established for infecting wild-caught *C. imicola* with AHSV (Venter *et al.*, 1991; Venter *et al.*, in press) and consequently the influence of temperature on AHSV transmission by *C. imicola* can now be studied. The present study was therefore carried out to determine the effect of temperature on vector competence and duration of the EIP in *C. imicola* infected with AHSV, as well as to establish the minimum temperature for AHSV replication.

4.3 METHODS

Virus

Of the 4 AHSV serotypes that *C. imicola* have so far been infected with in laboratory trials, serotype 8 has the highest infection prevalence, with 28% of midges infected after 10 days at 23.5°C (Venter *et al.*, 1991; Venter *et al.*, in press). Consequently, to avoid having to use unreasonably large numbers of *C. imicola* to determine the effect of temperature on AHSV replication, AHSV8 was used in this study.

To maximise the likelihood that *C. imicola* would ingest sufficient virus to initiate infection, it was essential to use a high titre of virus. To produce a high viral titre, AHSV8 was initially propagated in monolayers of baby hamster kidney (BHK-21) cells. These cells were grown in 175 cm^2 flasks and overlaid with 25 ml of Glasgow Minimum Essential Medium (MEM; Life Technologies). When the cells displayed 100% cytopathic effect (CPE) (after ≈3 days at 37°C), the virus suspension was removed and centrifuged at 1500g for 5 minutes. The pelleted cells were then resuspended in 5 ml of the supernatant and kept overnight at –70°C, while the remaining supernatant was held at 4°C. The cell suspension was subsequently thawed slowly, resulting in the release of virus from the cells. This was then mixed with 20 ml of the original supernatant and centrifuged at 1500g for 5 minutes. The resulting supernatant was then separated from the cell pellet and kept at 4°C until required for the feeding trials.

Insects

Adult *Culicoides* were collected nightly at the Onderstepoort Veterinary Institute (OVI; 25°39'S, 28°11'E), South Africa, between January and March 1999. *Culicoides imicola* is the predominant *Culicoides* species at this site and occurs in large numbers during the summer (Venter *et al.*, 1996). *Culicoides* were caught near a horse stable, using 220V down-draught suction light-traps equipped with 8W UV-light tubes and collected into 500 ml plastic beakers. Mosquito netting was placed around the traps to prevent the capture of larger insects and crumpled paper towel was placed in the beakers prior to catching to provide shelter for the trapped midges from the down-draught of the trap.

The catches were collected each morning before sunrise and emptied into black boxes (450 x 330 x 280 mm). A white translucent funnel was placed over a hole on one side of the box. Adult midges moved through the funnel towards the light and were collected in 300 ml unwaxed cardboard cups with fine mesh tops. Adults were then kept at 23.5°C (for a minimum of two days) until blood-feeding and provided with a 10% sucrose solution medicated with antibiotics (penicillin/streptomycin). The midges were deprived of sugar solution 24 hours before the blood-meal to maximise their feeding rate.

Feeding technique

Adults were blood-fed through a 1-day-old chicken skin membrane using the artificial feeding apparatus described by Venter *et al.* (1991; Figure 4.1). Blood-meals were taken from a suspension consisting of 9 ml of defibrinated horse blood mixed with 1 ml of AHSV8 solution, producing a final titre of ≈6 $\log_{10}$ $TCID_{50}$/ml. Up to 10 batches of *Culicoides* per day were fed on the blood-virus suspension and to confirm that there was no loss of infectivity during the day, 0.5 ml blood samples were taken immediately before and after the feeding process. These samples were stored in 1.5 ml Eppendorf tubes at –70°C until virus isolation could be carried out.

During feeding the midges were kept at 23.5°C, 50-70% relative humidity and 1% daylight, while the blood-virus suspension was maintained at 37°C. Midges were allowed to feed for 30-45 minutes, after which time they were immobilised by placing them at –10°C for a few minutes and then transferred to a refrigerated chill table. Fully engorged females were placed into 300 ml unwaxed cardboard cups with fine mesh tops (about 100 individuals/cup). A pad of cotton wool soaked in 10% sucrose solution medicated with antibiotics was placed on the mesh and replaced daily.

Virus-fed females were then kept at constant temperatures of either 15, 18, 26 or 30°C. Samples consisting of 4 pools of 25 *C. imicola* (to determine the effect of temperature on virus development) and 100 individual *C. imicola* (to determine the proportion of *C. imicola* that were capable of viral transmission and the duration of the EIP) were taken immediately after the blood-meal and then every 5 days for 25 days at 15°C, every 4 days for 20 days at 18°C, and every 2 days for 10 days at 26 and

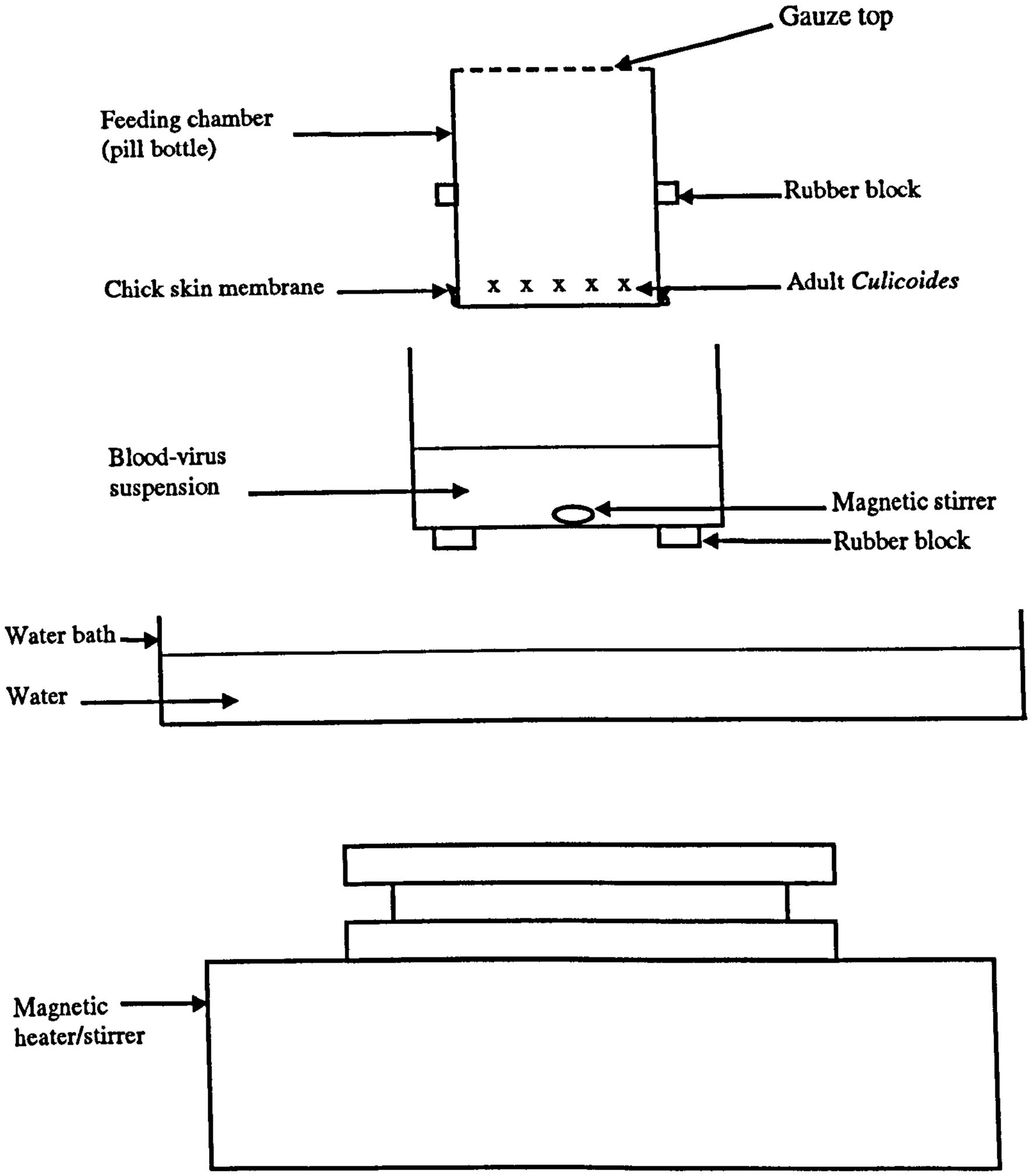


Figure 4.1 Blood-feeding apparatus. The feeding chamber consists of a plastic pill bottle, with the top replaced by gauze and the bottom replaced by a chick skin membrane. Adult *Culicoides* are placed into the feeding chamber, which is then placed into a second wider pill bottle, containing the blood-virus suspension and a magnetic stirrer. The two containers are placed into a water bath (an aluminium foil dish) on a magnetic heater/stirrer. The blood-virus suspension is heated to 37°C and is mixed slowly with the magnetic stirrer. Female *Culicoides* blood-feed through the chick skin membrane. (Adapted from Venter *et al.*, 1991).

30°C. Midges were sorted to species level on the chill table and the *C. imicola* samples were placed into 1.5 ml Eppendorf tubes and stored at –70°C until virus isolation could be carried out.

Virus titration assay

Midge samples were ground up using motor-driven 1.5 ml polypropylene pestles (Anachem), in 1ml of MEM that contained 200 µg/ml of penicillin/streptomycin and 2.5 µg/ml of fungizone. In the case of pools of *C. imicola*, 200 µl of the suspension was subsequently diluted 1 in 10 in MEM and passed through a 200 nm syringe filter (Supor^R Acrodisc^R 32; Gelman Sciences) to remove bacteria and fungi, but not AHSV8 virions which are ≈70 nm in diameter. Three additional ten-fold dilutions were then prepared. Dilutions of individual *C. imicola* were not prepared at this stage, since it was necessary to first establish which individuals contained virus, as only a small proportion were likely to be infected.

Assays were carried out in 96-well microtitre plates. Each well contained a monolayer of BHK-21 cells and 100 µl of MEM supplemented with 2% foetal calf serum, 100 µg/ml of penicillin/streptomycin and 2.5 µg/ml of fungizone. One hundred microlitres of each midge suspension and dilution (in the case of pools of *C. imicola*) were inoculated onto each of 4 microtitre plate wells. The inoculated microtitre plates were subsequently incubated at 37°C in a carbon dioxide incubator. After five days the plates were observed microscopically for CPE which was used as a positive indicator of the presence of virus. If virus was detected in individual *C. imicola*, 2 ten-fold dilutions of the original suspension were prepared and inoculated on to BHK-21 cells, as described above. Viral titres were calculated using a method adapted from Spearman (1908) and Kärber (1931) and expressed as $TCID_{50}$/25 midges for pools of *C. imicola* (Appendix 1.3) or $TCID_{50}$/midge for individual *C. imicola* (Appendix 1.4). The assay could detect viral titres of 0.75-5.5 $\log_{10}$ $TCID_{50}$/25 midges and 0.75-3.5 $\log_{10}$ $TCID_{50}$/midge. Samples with <0.75 $\log_{10}$ $TCID_{50}$ were classed as negative.

Seven ten-fold dilutions of the blood samples were also prepared and 100 µl of each dilution was inoculated onto each of 6 microtitre plate wells containing BHK-21 cells.

Viral titres of 1.67-8.5 $\log_{10}$ $TCID_{50}$/ml could be detected and were determined as described above.

Field controls

AHSV and other orbiviruses (e.g. bluetongue virus; BTV) are endemic in the Onderstepoort area. Hence it is possible that *C. imicola* females, which had taken a blood-meal prior to being trapped, may have already been infected with a virus. To assess the likelihood that the virus detected in *C. imicola* was acquired from the feeding trials and not from the field, it was necessary to determine the prevalence of field infections in the OVI *C. imicola* population. To do this, collections of *Culicoides* were made in phosphate buffered saline with 0.5% Savlon and parous *C. imicola* females (i.e. females that have taken a blood meal and laid at least one batch of eggs), identified by the presence of a red pigment in the abdomen (Dyce, 1969), were subsequently sorted into 15 pools of 100. These samples were then processed for virological assay as described for the pools of 25 midges.

Vector competence

Initial conditions: To ensure that individual *C. imicola* tested at the different temperatures started with the same initial conditions, that is the proportion of *C. imicola* that ingested virus from the blood-virus suspension and the titre of virus they ingested, it was necessary to compare the values for these traits after feeding (day 0 values). The proportion data were analysed using a 1-way analysis of variance (ANOVA) with binomial errors (for proportion data; Crawley, 1993). The log transformed viral titres in the positive midges were compared using a 1-way ANOVA with normal errors.

Infection rate: This refers to the proportion of *C. imicola* which were susceptible to infection with AHSV8 (i.e. proportion of midges in which virus could replicate in the midgut). Typically after ingestion of a viraemic blood-meal, the viral titre per midge declines during an 'eclipse phase', but subsequently rises as the virus starts replicating in the midgut. Hence to determine the effect of temperature on the infection rate, the proportion of *C. imicola* that tested positive for AHSV8 (i.e. titre ≥ 0.75 $\log_{10}$

$TCID_{50}$/midge) from the end of the eclipse phase onwards at each temperature were compared using a 1-way ANOVA with binomial errors.

Vector competence: The proportion of *Culicoides* that are capable of virus transmission may be lower than the proportion that can become infected, as some individuals may possess a midgut escape barrier (where virus is restricted to the midgut; Jennings and Mellor, 1987) or a dissemination barrier (where virus is unable to infect secondary target organs; Fu *et al.*, 1999). *Culicoides imicola* with a AHSV8 titre ≥2 $\log_{10}$ $TCID_{50}$/midge after the eclipse phase were considered to be capable of transmitting virus. This value was taken as Jennings and Mellor (1987) found that only individual *C. variipennis sonorensis* (from the Pirbright colony; Boorman, 1974) containing ≥2.5 $\log_{10}$ $TCID_{50}$ of BTV (a virus closely related to AHSV) transmitted virus. Since *C. imicola* are smaller than *C. variipennis sonorensis* it is unlikely that virus will have to replicate to such a high titre before they will be capable of transmission. *Culicoides imicola* females from the OVI population (mean wing length 0.97 mm) are 2/3 size of *C. variipennis sonorensis* females from the Pirbright colony (mean wing length 1.45 mm). Consequently, if we assume that the volume of a *C. imicola* female is only 8/27 of that for a *C. variipennis sonorensis* female, it is reasonable to infer that virus will only have to replicate to a titre of 2 $\log_{10}$ $TCID_{50}$/midge (2.5 $\log_{10}$ = 316; 316 x 8/27 = 94; 94 = 2 $\log_{10}$) before *C. imicola* are capable of transmission. Hence to determine the effect of temperature on vector competence, the proportion of *C. imicola* with AHSV8 titre ≥2 $\log_{10}$ $TCID_{50}$/midge from the end of the eclipse phase onwards at each temperature were compared using a 1-way ANOVA with binomial errors.

Extrinsic incubation period

The EIP was defined as the interval between the viraemic blood-meal and when AHSV8 had replicated to a titre of ≥2 $\log_{10}$ $TCID_{50}$/midge. The median time required for *C. imicola* to complete the EIP was calculated at each temperature. To do this the time-cumulative proportion of individual *C. imicola* with viral titre ≥2 $\log_{10}$ $TCID_{50}$/midge (after the eclipse phase) at each temperature was transformed using probit analysis (used to linearise cumulative curves) and plotted against the logarithm

of days. Linear regression was then used to fit the best straight line and from this an estimate of the median EIP was obtained (see Figure 3.2).

Extrinsic incubation rates (EIR; reciprocal of the EIP) were then calculated and regressed as a linear function of temperature. The theoretical minimum temperature for AHSV8 replication in *C. imicola* could then be estimated by extrapolation of the regression line to the x-axis and the number of day degrees above the minimum temperature that were required for AHSV8 development was estimated as the reciprocal of the slope of the line (see Figure 3.2).

4.4 Results

Virus development

The geometric mean titre of AHSV8 in pools of *C. imicola* incubated at different temperatures are shown in Figure 4.2. At each temperature, the mean AHSV8 titre declined after the blood-meal (eclipse phase), but subsequently increased over time. This pattern is typical of arbovirus development in a vector and is related to initial digestion and/or excretion of virus particles as well as the uncoating of virus particles during infection of the midgut cells (core particles are less infectious for BHK-21 cells than intact virus particles; Mertens *et al.*, 1996), followed by replication of virus in the midgut cells and secondary target organs. However, temperature affected both the rate of AHSV8 development and the extent of replication. For example, at 26°C replicating virus was detected by day 6 and AHSV8 titres of ≈3.4 $\log_{10}$ $TCID_{50}$/25 midges were subsequently observed, whereas at 15°C replication of AHSV8 was not detected until day 15 and viral titres of only 2.4-2.7 $\log_{10}$ $TCID_{50}$/25 midges were subsequently obtained.

Vector competence

The proportion of individual *C. imicola* that ingested AHSV8 from the blood-virus suspension ($\chi^2 = 1.98$, df = 3, NS) and the titre of AHSV8 ingested by these midges ($F_{3,46} = 0.06$, NS) did not differ significantly among the incubation temperatures (day 0 values; Figure 4.3, blue and red lines). Overall 42% of *C. imicola* ingested detectable

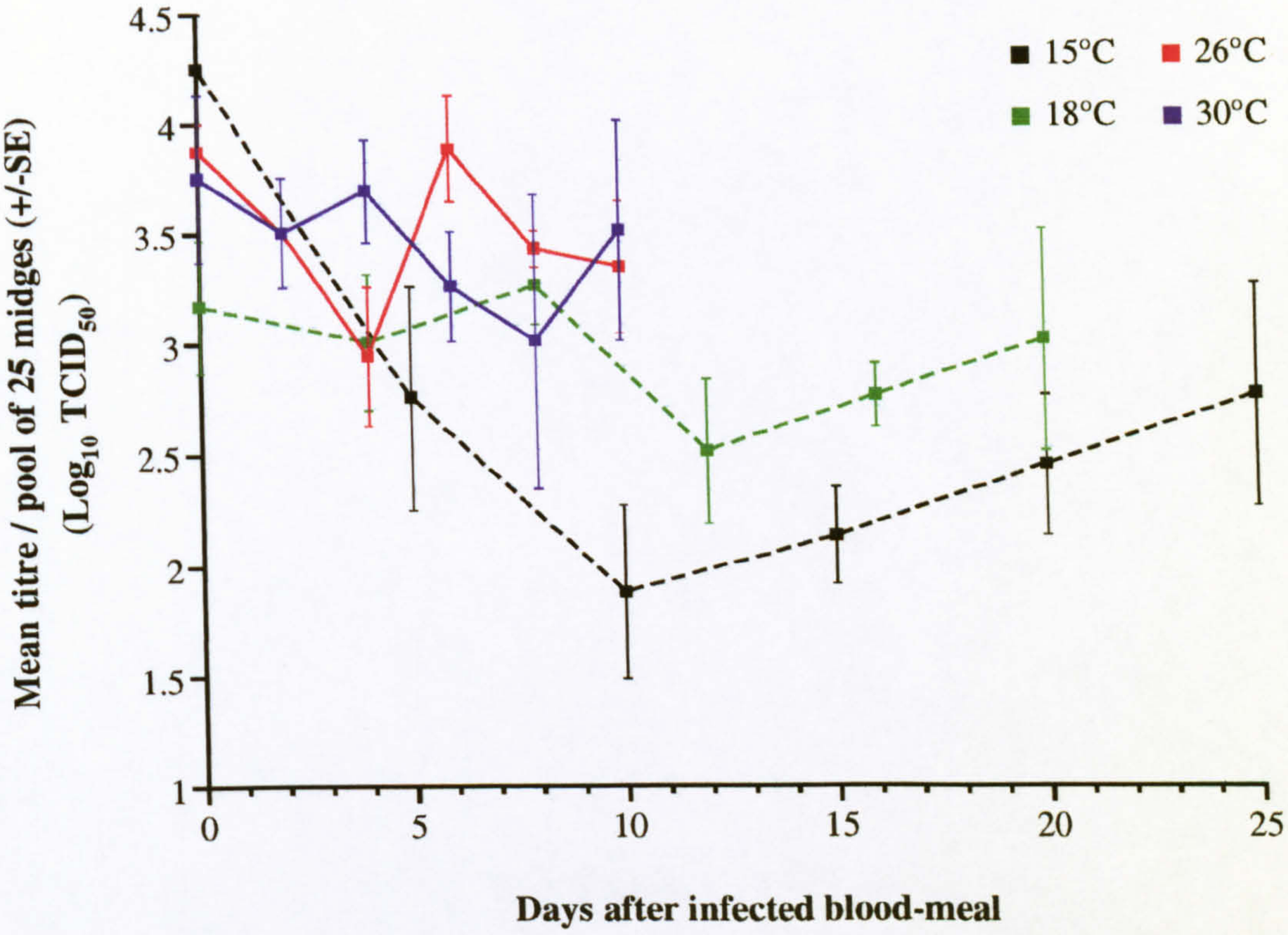


Figure 4.2 Geometric mean titre of African horse sickness virus serotype 8 in pools of 25 *C. imicola* incubated at different temperatures (based on 4 pools of 25 midges per test day).

titres of AHSV8 from the blood-virus suspension and the geometric mean viral titre ingested by these midges was 1.6 $\log_{10}$ $TCID_{50}$/midge.

The end of the eclipse phase was signalled by an increase in the geometric mean titre per positive midge after the initial decline in titre and was considered to be day 10 at 15°C, day 8 at 18°C, day 6 at 26°C and day 4 at 30°C (Figure 4.3, red lines). The proportion of *C. imicola* with AHSV8 titre ≥0.75 $\log_{10}$ $TCID_{50}$/midge from the end of the eclipse phase onwards (i.e. infection rate) was significantly higher at 26°C than at the other temperatures ($\chi^2 = 115.9$, df = 1, $p<0.01$) (Figure 4.3, blue lines). However, temperature did not significantly affect the vector competence of *C. imicola* for AHSV8 ($\chi^2 = 4.64$, df = 3, NS) and overall only 2% of *C. imicola* from the eclipse phase onwards had a viral titre ≥2 $\log_{10}$ $TCID_{50}$/midge (Figure 4.3, green lines).

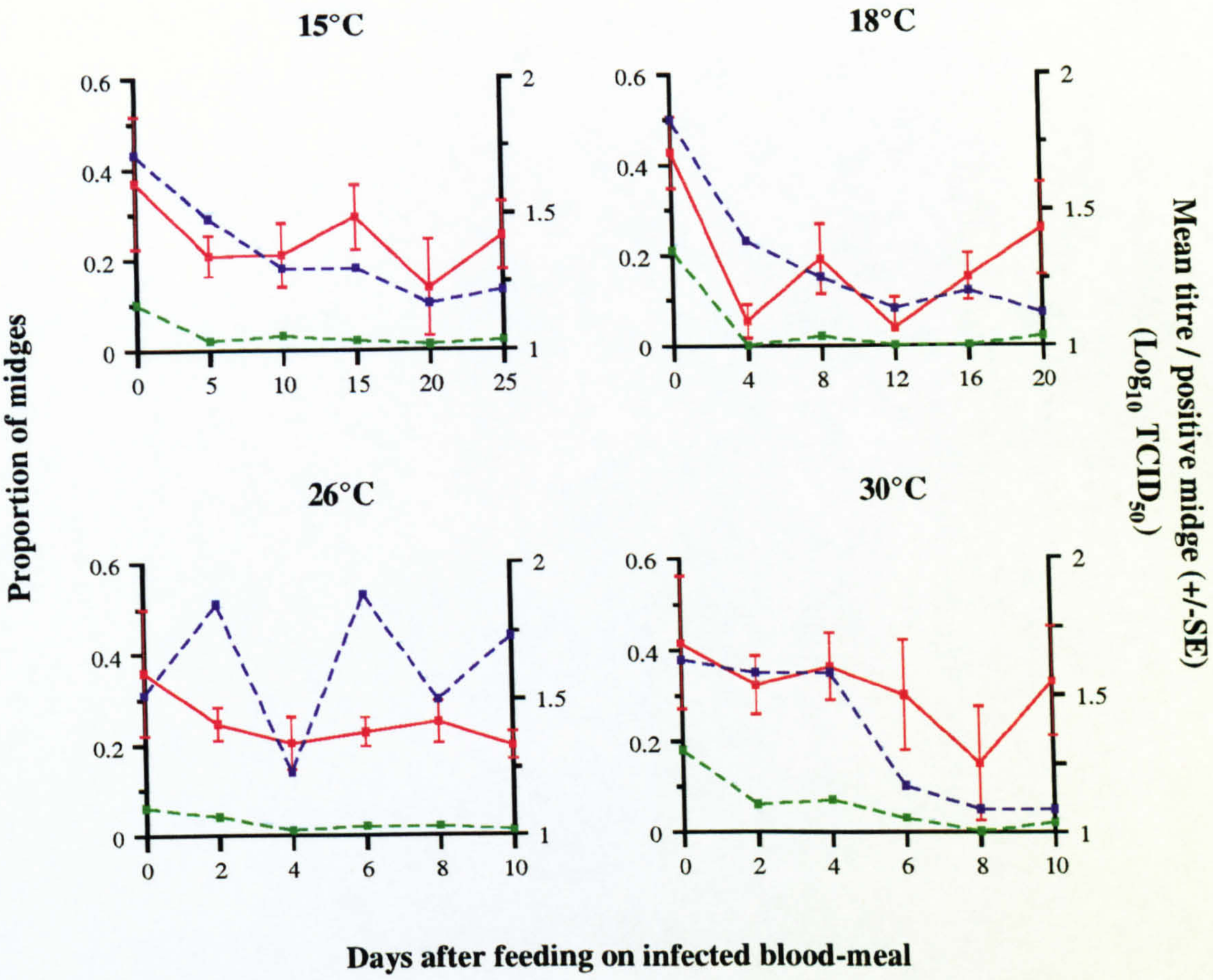


Figure 4.3 Effect of temperature on the proportion of individual *C. imicola* that tested positive for AHSV8 (i.e. vial titre ≥0.75 $\log_{10}$ $TCID_{50}$/midge; blue lines) and on the proportion of *C. imicola* with AHSV8 titre ≥2 $\log_{10}$ $TCID_{50}$/midge (green lines) at intervals after the infected blood-meal (based on 100 individuals per test day). Also shown are the geometric mean AHSV8 titres per positive midge (red lines).

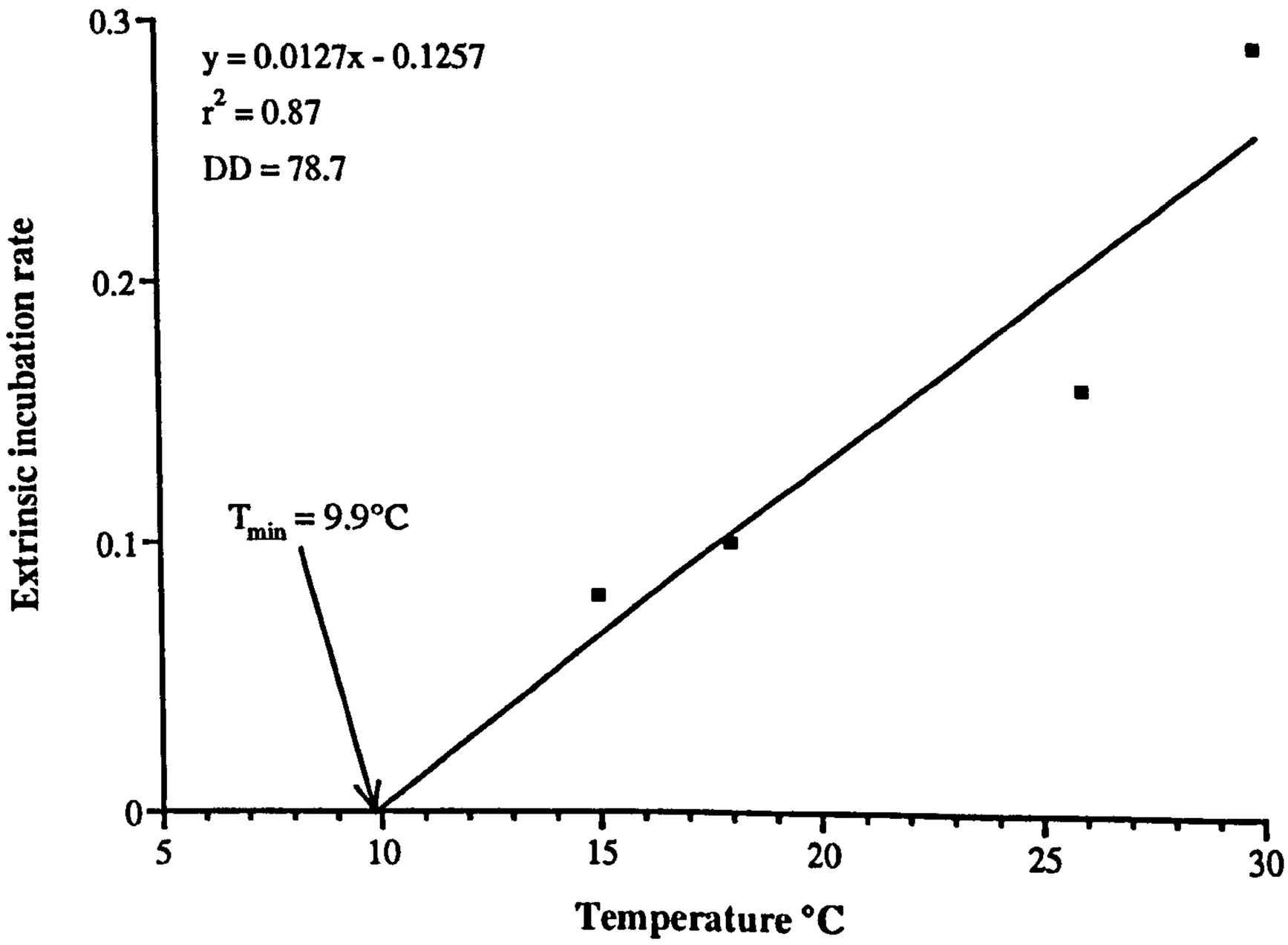
Extrinsic incubation period

The estimated duration of the median EIPs for AHSV8 in *C. imicola* maintained at the different temperatures are shown in Table 4.1. The rate of AHSV8 development (1/EIP) in *C. imicola* increased with temperature (Figure 4.4). The theoretical minimum temperature for AHSV8 development was estimated to be 9.9°C and 78.7 day degrees were required above this threshold temperature for virus development.

Table 4.1 Estimated duration of the median EIP for AHSV8 in *C. imicola* maintained at different temperatures.

Temperature	15°C	18°C	26°C	30°C
EIP - days	12.4	10.0	6.4	3.4

Figure 4.4 Extrinsic incubation rates (1/median extrinsic incubation period) for AHSV8 in *C. imicola* maintained at different temperatures. T_{min} is the theoretical minimum temperature for virus development and DD is the total number of day degrees required above T_{min} for virus development.



Blood-meal and field controls

AHSV8 titres detected in the blood-virus suspension before and after feeding did not differ significantly ($F_{1,20} = 3.6$, NS). Virus was not detected in any of the pools of field collected parous *C. imicola.*

4.5 DISCUSSION

The vector competence of the Onderstepoort *C. imicola* females for AHSV8 did not vary significantly among the incubation temperatures and overall only 2% of females tested were considered capable of transmitting virus. However AHSV8 development in *C. imicola* was affected by temperature and the duration of the EIP was shorter at higher temperatures. The minimum temperature for AHSV8 development in *C. imicola* was estimated to be 9.9°C.

There are two potential confounding factors in this study. First, the wild-caught *C. imicola* may have been infected with virus in the field. However, since virus was not detected in the control pools of parous *C. imicola*, it is unlikely that virus recovered from the laboratory-infected midges was acquired from the field. Second, the minimum viral titre a midge must contain before it is considered capable of virus transmission has yet to be established for *C. imicola*. We considered that *C. imicola* with viral titres ≥2 $\log_{10}$ $TCID_{50}$/midge were likely to transmit virus. However, although this figure is hypothetical, it does appear to be reasonable when considered in terms of the range of AHSV titres observed in *C. imicola*. Additionally, in other *Culicoides* vector species, not all individuals develop a large enough viral titre for them to be considered as competent vectors. For example, Fu *et al.* (1999) found that only the small proportion of *C. variipennis sonorensis* with the largest viral titres would ever be capable of transmitting BTV. Nevertheless, it is critical both for future work and for the interpretation of data such as that presented here, that a better understanding of the threshold titre for *C. imicola* vector competence is obtained.

It is perhaps surprising that temperature did not affect the vector competence of *C. imicola* for AHSV8, since vector competence increased linearly between 15 and 30°C in trials involving *C. variipennis sonorensis* and AHSV4 (Chapter 3). However, the effect of temperature on vector competence of *Culicoides* vectors for orbiviruses

seems to be variable, and in the same trials it was found that temperature had no effect on the proportion of *C. variipennis sonorensis* capable of transmitting BTV10 or BTV16. Therefore, even though temperature did not influence the vector competence of *C. imicola* for AHSV8, it does not necessarily follow that this will also be the case with other AHSV serotypes or virus species. In addition, other populations of *C. imicola* may vary in their susceptibility to AHSV8 and in their response to temperature.

The low vector competence of the Onderstepoort *C. imicola* females for AHSV8 may have been partially due to the fact that less than half of the midges tested after feeding had actually ingested detectable titres of virus. However, given that the blood-virus suspension contained an AHSV8 concentration of 6 $\log_{10}$ $TCID_{50}$/ml and that the blood-meal size for *C. imicola* is between 0.03 and 0.07 µl (Hamblin, pers. comm.), individual midges should have ingested between 1.5 and 1.8 $\log_{10}$ $TCID_{50}$ of virus, which is well within the range of the assay system used in this study. There are two possible reasons why some midges did not ingest virus at a detectable level. First, the midges may have ingested smaller blood-meals (i.e. <0.03 µl), reducing their chances of acquiring virus. Second, the virus particles may not have been homogeneously distributed throughout the blood-virus suspension, resulting in some midges only ingesting blood. In *C. variipennis sonorensis*, a species which takes in up to three times the volume of blood per blood-meal as *C. imicola* (Mellor, 1990), all females that feed on a blood-virus suspension with a viral titre of 6 $\log_{10}$ $TCID_{50}$/ml are found to have ingested virus. This result suggests that the size of the blood-meal may be an under-rated aspect of vector competence.

The fact that not all blood-engorged females were found to have ingested AHSV8 suggests that we may be underestimating the proportion of competent *C. imicola* in the population. If we include all the females tested from the end of the eclipse phase onwards in the analysis we find that ≈2% were vector competent, yet some of these females may not have initially ingested virus. Since virus was only detected in 42% of females after blood-feeding, it is possible that actually ≈5% of the population were competent to transmit AHSV8. However, although the vector competence of the Onderstepoort *C. imicola* females for AHSV8 was low, regardless of which figure we

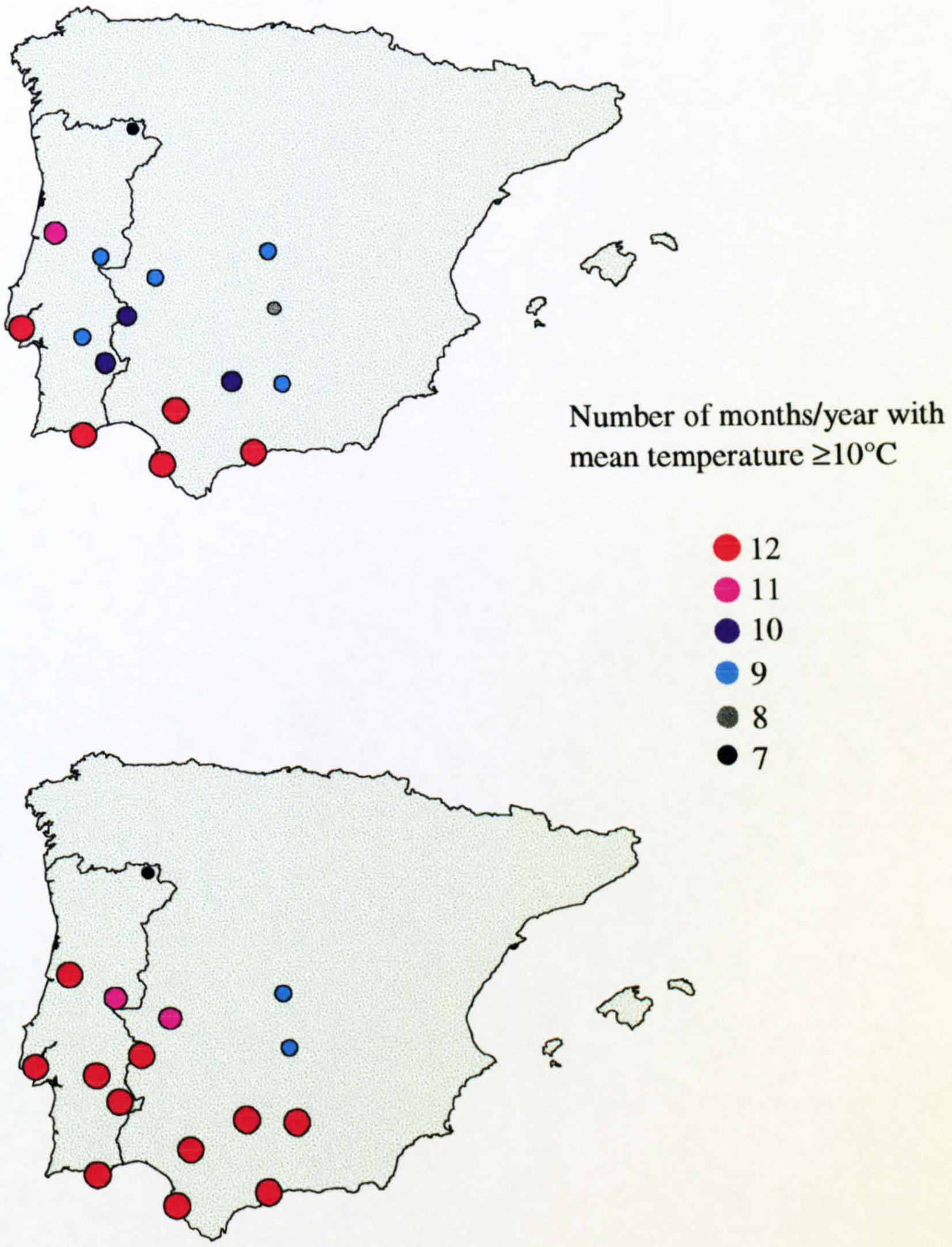
accept, this will be compensated for by the potentially huge population sizes of *C. imicola*. For example, Meiswinkel (1998) caught over a million *C. imicola* in a single light trap in a single night at OVI during March 1996.

The duration of the AHSV8 EIP in *C. imicola* was shorter at high temperatures, indicating that females would be able to transmit virus sooner when conditions are warm. Temperature also has a similar effect on the EIP in other *Culicoides*/virus combinations that have been investigated (e.g. *C. variipennis sonorensis* infected with AHSV4, AHSV9, BTV10, BTV11, BTV16 or epizootic haemorrhagic disease virus serotype 1; Mullens *et al.*, 1995; Wellby *et al.*, 1996; Chapter 3). In addition, the theoretical minimum temperature for AHSV8 development in *C. imicola* of 9.9°C is similar to estimates of the minimum temperature for orbivirus development in *C. variipennis sonorensis* (Chapter 3).

Given these findings, if the global mean temperature does increase by 2°C during the next 100 years (as predicted by climate change scenarios; Intergovernmental Panel on Climate Change, 1996), it is likely that virus replication in *C. imicola* will become more rapid and will be able to occur over greater periods of the year, especially in regions where low temperatures during winter currently restrict virus development. For example, at 16 sites within the *C. imicola* distribution in Iberia, virus development could currently occur over 7-12 months of the year (depending on the site), when the mean monthly temperature is ≥10°C (Figure 4.5a). However, with a 2°C increase in temperature, virus development could potentially occur over 12 months of the year at the majority of these sites (Figure 4.5b). In this analysis I used the mean monthly temperatures for each of the sites (calculated from Anon, 1972). However, it must be borne in mind that virus development may also occur in months in which the mean temperature is <10°C, but where temperatures exceed this threshold for a number of hours per day. Nevertheless, while these projections may be crude, they do point towards one of the potential impacts of climate change on AHSV transmission.

However, to determine the effect of temperature on AHSV transmission by *C. imicola*, the impact of temperature on vector survival and the blood-feeding interval, as well as on vector competence and the EIP must be considered.

a.



b.

Figure 4.5 Number of months per year, at sites within the *C. imicola* distribution in Iberia, with a mean monthly temperature ≥10°C (i.e. number of months over which AHSV8 development could occur), both (a) currently and (b) if temperatures increase by 2°C.

The relationship between these factors can be described as follows:

$M = Vp^n$

where M = proportion of midges that could take at least one virus transmissive blood-meal (after initially ingesting a viraemic blood-meal), V = proportion of competent midges, p = daily survival rate and n = days to first virus transmissive blood-meal (i.e. first blood-meal after the EIP). However, one of the limitations of working with wild-caught *Culicoides* is that their age and background are unknown. Quantifying the effect of temperature on vector survival is therefore difficult, since these factors may confound the analysis. Determining the blood-feeding interval (which is largely governed by the time required for the eggs to develop) at different temperatures can also be difficult, since wild-caught midges are reluctant to oviposit under laboratory conditions.

Nevertheless while the impact of temperature on survival and blood-feeding may be difficult to determine, the daily survival rate and the blood-feeding interval for a particular field population of *Culicoides* can be estimated. For example, Baylis (pers. comm.) found that the daily survival rate of *C. imicola* during March at OVI was 0.74 and that females appear to blood-feed on average every 3 days. This information together with that established in this study (i.e. V = 0.02 and n = 6; given that the mean temperature at OVI during March is 23.4°C, *C. imicola* would complete the EIP after 5.8 days and take their first virus transmissive blood-meal after 6 days) can then be used to calculate the proportion of Onderstepoort *C. imicola* females that could transmit AHSV8. Consequently, one *C. imicola* for every 304 *C. imicola* that ingest a blood-meal containing AHSV8 could potentially transmit virus.

In this chapter, I have presented the results of the first study of how temperature influences the transmission of AHSV by the field vector *C. imicola*. This is a considerable improvement on previous work in this area, which has involved the use of laboratory strains of *C. variipennis sonorensis*. However, one of the major disadvantages of working with wild-caught *Culicoides* is that it is difficult to determine the effect of temperature on vector survival and the blood-feeding interval, both of which are important factors influencing virus transmission. Nevertheless, while it must

be borne in mind that different geographical populations of a species may vary in their susceptibility to a virus and in their response to temperature, this study has provided a useful insight into the impact that temperature can have on AHSV transmission by *C. imicola* and can be used to assess the risk of AHSV transmission occurring within a particular region, both currently and if conditions should warm with climate change.

CHAPTER 5: HIGHER IMMATURE REARING TEMPERATURES INDUCE VECTOR COMPETENCE FOR BLUETONGUE VIRUS AND AFRICAN HORSE SICKNESS VIRUS IN *CULICOIDES NUBECULOSUS* (DIPTERA: CERATOPOGONIDAE).

5.1 ASTRACT

Culicoides nubeculosus, which is found throughout much of Europe, is generally considered to be incapable of transmitting bluetongue virus (BTV) and African horse sickness virus (AHSV). This is thought to be the result of a midgut infection barrier that prevents the viruses from entering the haemocoel. However, when the immature stages were reared at 33°C, the oral infection rate of *C. nubeculosus* for both BTV serotype 10 and AHSV serotype 4 was significantly higher than when they were reared at temperatures between 25 and 32°C. This marked increase in susceptibility of *C. nubeculosus* to the viruses occurred as the upper lethal rearing temperature of 35°C was approached. One possible explanation is that the integrity of the gut wall was damaged by the extreme conditions, thereby allowing the virus particles to bypass the gut barriers and enter the haemocoel. The critical period, when exposure to hot conditions could induce competence for the viruses, appeared to be during the development of the adult midgut epithelial cells i.e. from before the larval/pupal moult to the end of the pupal stage. This phenomenon could result in the occurrence of competent *C. nubeculosus* in the field, especially considering the increase in frequency and intensity of extremely hot days that are predicted to occur with climate change and could result in greater areas of Europe being at risk of BTV and AHSV.

5.2 INTRODUCTION

Bluetongue virus (BTV) and African horse sickness virus (AHSV) are both dsRNA viruses within the genus *Orbivirus* of the family Reoviridae and 24 BTV serotypes and 9 AHSV serotypes are currently recognised. BTV infects all species of ruminants, causing severe disease in certain breeds of sheep (MacLachlan, 1994) and in some species of deer (Robinson *et al.*, 1967; Stair *et al.*, 1968). AHSV infects equids and the disease is most devastating in horses, with mortality ranging from 70 to 95% (Coetzer and Erasmus, 1994). BTV occurs between latitudes of approximately 40°N to 35°S (Mellor, 1990; Mellor and Boorman, 1995; Mellor, 1996), while AHSV is usually confined to sub-Saharan Africa (Mellor, 1994). However, there have also been

sporadic outbreaks of both viruses in southern Europe (Campano Lopez and Sanchez Botija, 1958; Diaz Montilla and Panos Marti, 1968; Vassalos, 1980; Dragonas, 1981; Rodriguez *et al.*, 1992; Anon, 1998; Anon, 1999b,c,d,e; Anon, 2000b).

BTV and AHSV are transmitted between their respective vertebrate hosts by biting midges of the genus *Culicoides*. Although there are estimated to be 1210 species of *Culicoides* in the world (Borkent and Wirth, 1997), only 24 species have so far been associated with BTV (see Table 1.1), while just 6 have been linked with AHSV (see Table 1.2). In addition, for these putative vector species viral susceptibility may vary among geographical populations (Jones and Foster, 1978) and among individuals within a population (Jennings and Mellor, 1987).

The ability of *Culicoides* to transmit orbiviruses (vector competence) is determined primarily at the midgut wall, which consists of a single layer of epithelial cells resting on a basement membrane. After taking a blood-meal from a viraemic vertebrate host, blood and virus are deposited into the posterior region of the insect's midgut. In competent individuals, virus particles attach themselves to the luminal surface of the gut cells, infect these cells and replicate in them. Progeny virions are then released through the basement membrane to the haemocoel where they infect secondary target organs including the salivary glands. After replication in the salivary glands, viral transmission can occur during subsequent biting activity (Mellor, 1990). However in refractory individuals, one of two barriers may exist in the midgut: a midgut infection barrier, where virus is unable to enter the midgut cells or a midgut escape barrier, where virus can replicate in the midgut cells but is unable to exit into the haemocoel (Jennings and Mellor, 1987). Fu *et al.* (1999) have also reported the existence of a dissemination barrier, where virus that enters the haemocoel is unable to infect secondary target organs. These barriers are under genetic control (Jones and Foster, 1974; Jones and Foster, 1978; Tabachnick, 1991).

In addition to genetic mechanisms, extrinsic factors may be involved in determining vector competence of haematophagous insects to arboviruses. For example, larval nutrition (Grimstad and Haramis, 1984; Grimstad and Walker, 1991; Zhang *et al.*, 1993; Nasci and Mitchell, 1994), larval rearing temperature (Kay *et al.*, 1989; Hardy *et al.*, 1990; Turell, 1993), extrinsic incubation temperature (temperature of viral

replication; Takahashi, 1976; Cornel *et al.*, 1993; Turell *et al.*, 1985; Lundström *et al.*, 1990; Turell and Lundström, 1990; Mullens *et al.*, 1995; Wellby *et al.*, 1996) and concurrent infection with virus and a parasite (Mellor and Boorman, 1980; Turell *et al.*, 1984; Turell *et al.*, 1987; Paulson *et al.*, 1992; Zytoon *et al.*, 1993; Vaughan and Turell, 1996a,b) have been shown to modify vector competence in various vector/arbovirus systems.

In the extreme, extrinsic factors can result in genetically non-competent individuals becoming phenotypically competent. Such is the case with the northern European midge *C. nubeculosus*, which is generally considered to be incapable of transmitting BTV or AHSV due to a midgut infection barrier (Mellor *et al.*, 1975; Mellor and Boorman, 1980). However, in a preliminary investigation, Mellor *et al.* (1998) showed that a 5-10°C rise in the larval rearing temperature, from the standard rearing temperature of 25°C, could increase the oral infection rate of *C. nubeculosus* for AHSV from <1% to >10%. They speculated that this phenotypic change could result from a 'leaky midgut', where virus leaks directly into the haemocoel, bypassing the midgut barriers. Once in the haemocoel the virus can replicate and be transmitted even by what is normally considered to be a non-vector species.

The implications of these findings are particularly worrying with regards to the risk of AHSV in Europe. The distribution of AHSV is limited to areas where there are competent vectors. *Culicoides imicola* is considered to be the principal vector in Europe (Mellor, 1990; Mellor, 1996) but has so far only been found in south-western Iberia (Rawlings *et al.*, 1997), mainland Greece (Patakakkis, unpub. obs.) and the Greek islands of Lesbos (Boorman and Wilkinson, 1983), Rhodes (Boorman, 1986), Chios, Kos, Samos (Mellor, pers. comm.) and Evia (Patakakkis, unpub. obs.). However, if environmental factors (i.e. high temperatures) resulted in Palearctic species being able to transmit virus where previously they were considered to be refractory, then more northerly regions of Europe will be at risk to AHSV. In addition, the probability of such an event actually occurring becomes more likely considering the increase in frequency and intensity of extremely warm days predicted to occur due to climate change (Houghton, 1997).

Given these implications, the present study was carried out to confirm the preliminary findings of Mellor *et al.* (1998) using larger sample sizes and to determine if vector competence for BTV in *C. nubeculosus* could also be induced by elevated rearing temperatures. With regards to the risk of disease in the more northerly parts of Europe, it was also important to determine the lowest rearing temperature at which vector competence could be induced. Furthermore, it was necessary to establish whether elevated rearing temperatures were required for the whole life cycle before adult *C. nubeculosus* became susceptible to the viruses or whether hot conditions for part of the life cycle could have the same effect. Evidence from Lepidoptera suggests that midgut epithelial cells present in the adults are formed a short time before the larval/pupal moult (Baldwin *et al.*, 1996). If *C. nubeculosus* development follows a similar pattern then it is possible that hot conditions during the pupal stage alone could also result in competent adults.

However, in order to transmit virus to vertebrate hosts, female *C. nubeculosus* must not only become susceptible to the virus but survive long enough to complete the viral extrinsic incubation period (EIP; development time of the virus in the vector). Consequently, to establish whether elevated rearing temperatures affected the survival of adult *C. nubeculosus*, longevity of adults reared at control and elevated temperatures was determined. Finally, preliminary trials were carried out to investigate whether there were morphological and ultrastructural differences between the midguts of *C. nubeculosus* reared at control and elevated temperatures which could account for any changes in vector competence.

5.3 Methods

Insects and virus

Freshly laid *C. nubeculosus* eggs from the Pirbright colony were reared through to adulthood at the standard rearing temperature of 25°C and at experimental temperatures of 30, 31, 32 and 33°C, using established methods (Boorman, 1974). In addition, to investigate the effect of hot conditions during the pupal stage alone, *C. nubeculosus* were initially reared from eggs to the beginning of the pupal stage at 25°C. Pupae, which were 0-8 hours old, were then transferred to 33°C for the

remainder of the pupal stage. After eclosion, adults from all of the groups were kept at 25°C for two days before being blood fed.

The viruses used were BTV serotype 10 and AHSV serotype 4, both originally obtained from Onderstepoort Veterinary Institute, South Africa. BTV10 had been passaged initially in embryonated hen eggs, followed by seven passages in baby hamster kidney (BHK-21) cells. AHSV4 had been passaged once in suckling mouse brains and four times in BHK-21 cells. The viruses were then stored as tissue culture supernatants at 4°C until required.

Adult *C. nubeculosus* were blood fed using the artificial feeding apparatus and method described by Mellor *et al.* (1974), except that a stretched parafilm membrane was used in place of a 1-day-old chicken skin membrane (see Figure 3.1). Blood-meals were taken from a blood-virus suspension consisting of 1 ml of heparinised horse blood mixed with 1 ml of BTV10 or AHSV4, producing a final titre of 5.7-6.7 $\log_{10}$ $TCID_{50}$/ml. Horse blood was used as the source of blood to increase the likelihood of midges becoming infected with the viruses (Marchi *et al.*, 1995; Burroughs, unpub. obs.). Midges were allowed to feed for about 30 minutes, after which time they were lightly anaesthetised with carbon dioxide and the fully engorged females were separated from individuals which had not fed. Engorged females were then placed into waxed cardboard pill boxes with fine mesh tops (about 100 individuals/box) and kept for a further 10 days at 25°C. A pad of cotton wool soaked in 10% sucrose solution medicated with 100 µg/ml of penicillin/streptomycin was placed on the mesh and replaced daily. This provided a source of energy as well as maintaining a high humidity and hence increased the likelihood of midges surviving the incubation period. A moistened filter paper disc in the base of the pill box provided sites for oviposition. After 10 days, surviving midges were anaesthetised with carbon dioxide, placed individually into 1.5 ml Eppendorf tubes and kept at -70°C until they could be assayed for virus.

Virus titration assay

About 100 midges from each rearing temperature were ground up individually, using motor-driven 1.5 ml polypropylene pestles (Anachem), in 0.5 ml of Glasgow Minimum Essential Medium (MEM; Life Technologies) which contained 200 µg/ml

of penicillin/streptomycin and 2.5 µg/ml of fungizone. Two hundred microlitres of the individual midge suspension was then diluted 1 in 10 in MEM and passed through a 200 nm syringe filter (Supor^R Acrodisc^R 32; Gelman Sciences) to remove bacteria and fungi, but not the BTV or AHSV virions which are about 70 nm in diameter. Two additional ten-fold dilutions were then prepared.

Virus titrations were carried out in 96-well microtitre plates. Each well contained a monolayer of BHK-21 cells and 100 µl of MEM supplemented with 2% foetal calf serum, 100 µg/ml of penicillin/streptomycin and 2.5 µg/ml of fungizone. One hundred microlitres of each dilution of the midge samples was then inoculated onto each of six microtitre plate wells. The inoculated microtitre plates were subsequently incubated at 37°C in a carbon dioxide incubator. After five days the plates were observed microscopically for cytopathic effects which were used as a positive indicator of the presence of virus. Viral titres were then calculated using method adapted from Spearman (1908) and Kärber (1931) and expressed as $TCID_{50}$/midge (Appendix 1.5). The assay could detect viral titres between 1.367 and 4.2 $\log_{10}$ $TCID_{50}$/midge. Midges with <1.367 $\log_{10}$ $TCID_{50}$ of virus were classed as negative.

The proportion of *C. nubeculosus* that tested positive for virus (i.e. titre ≥1.367 $\log_{10}$ $TCID_{50}$/midge) at the different rearing temperatures and for the two viruses was analysed using a 2-way analysis of variance (ANOVA) with binomial errors (for proportion data; Crawley, 1993). In addition, the log transformed viral titres present in midges that tested positive for BTV and AHSV, at rearing temperatures of 33°C for the whole life cycle or just the pupal stage were compared using a 2-way ANOVA with normal errors.

Survival of adult C. nubeculosus

Culicoides nubeculosus were reared at 25 and 33°C. One to two day old adults were allowed to feed on a blood-virus suspension consisting of heparinised horse blood mixed with BTV10. About 50 blood-fed females from each rearing temperature were placed into separate pill boxes and kept at 25°C. A saturated NaCl solution maintained the humidity at 75% (Winston and Bates, 1960). Midges were provided with a pad of cotton wool soaked in 10% sucrose solution medicated with antibiotics for one hour a day. This enabled the midges to feed but did not alter the humidity too

greatly. The number of midges that died each day was then counted until all the midges had died.

Survival analysis with exponential errors (to describe a Type II survivorship curve where the risk of death is independent of age) and a reciprocal link was carried out on the times to death, to determine the mean survival times and daily survival rates. The effect of rearing temperature on survival was assessed using a t-test.

Electron microscopy

Culicoides nubeculosus were reared at 25 and 33°C. Four to five day old females were allowed to feed on a blood-virus suspension consisting of heparinised horse blood mixed with BTV10, for 30 minutes. About one hour after the blood-meal and immediately prior to dissection groups of 2-3 fully-engorged females were chloroformed. Dissection was carried out in phosphate buffered saline under a dissecting microscope. Initially the legs were removed and then the head and prothorax were removed by cutting transversely between the prothorax and mesothorax. The terminal region of the abdomen was also removed by cutting transversely between the seventh and eighth abdominal segments. Finally the wings were removed. The aim was to leave the midgut intact inside the body, removing the head and tip of the abdomen so that the fixative could subsequently penetrate through the gut tissue. A preliminary trial had found that dissecting the midgut from the body disrupted the midgut cells, particularly in the case of midges reared at 33°C.

In addition, 4-5 day old adults, which had been reared at 25 and 33°C, but which had not been blood fed were dissected. The procedure was the same as above, except the abdomen was cut between the fifth and sixth abdominal segments, because when the gut is not distended with blood, it fills a smaller portion of the abdomen.

Ten individuals from each category were dissected. After dissection each body was immediately transferred to 2.5% glutaraldehyde in 0.1 M phosphate buffer for 24 hours at 4°C. The bodies were then washed twice in 0.1 M phosphate buffer and stored in buffer until post-fixation.

Post-fixation, carried out at Aberdeen University, was in 0.2% osmium tetroxide in 0.1 M sodium cacodylate buffer for 16-18 hours at room temperature. The bodies

were then washed in distilled water and dehydrated in an ethanol series (70-100%). After dehydration, the bodies were embedded in araldite (CY212) resin and ultrathin sections were cut on a ultramicrotome with a diamond knife. The sections were then placed on to 300 mesh copper grids, stained with uranyl acetate and lead citrate and viewed with a Philips 301 transmission electron microscope at 80 kV.

5.4 RESULTS

Rearing temperature and vector competence of C. nubeculosus *for BTV10 and AHSV4*

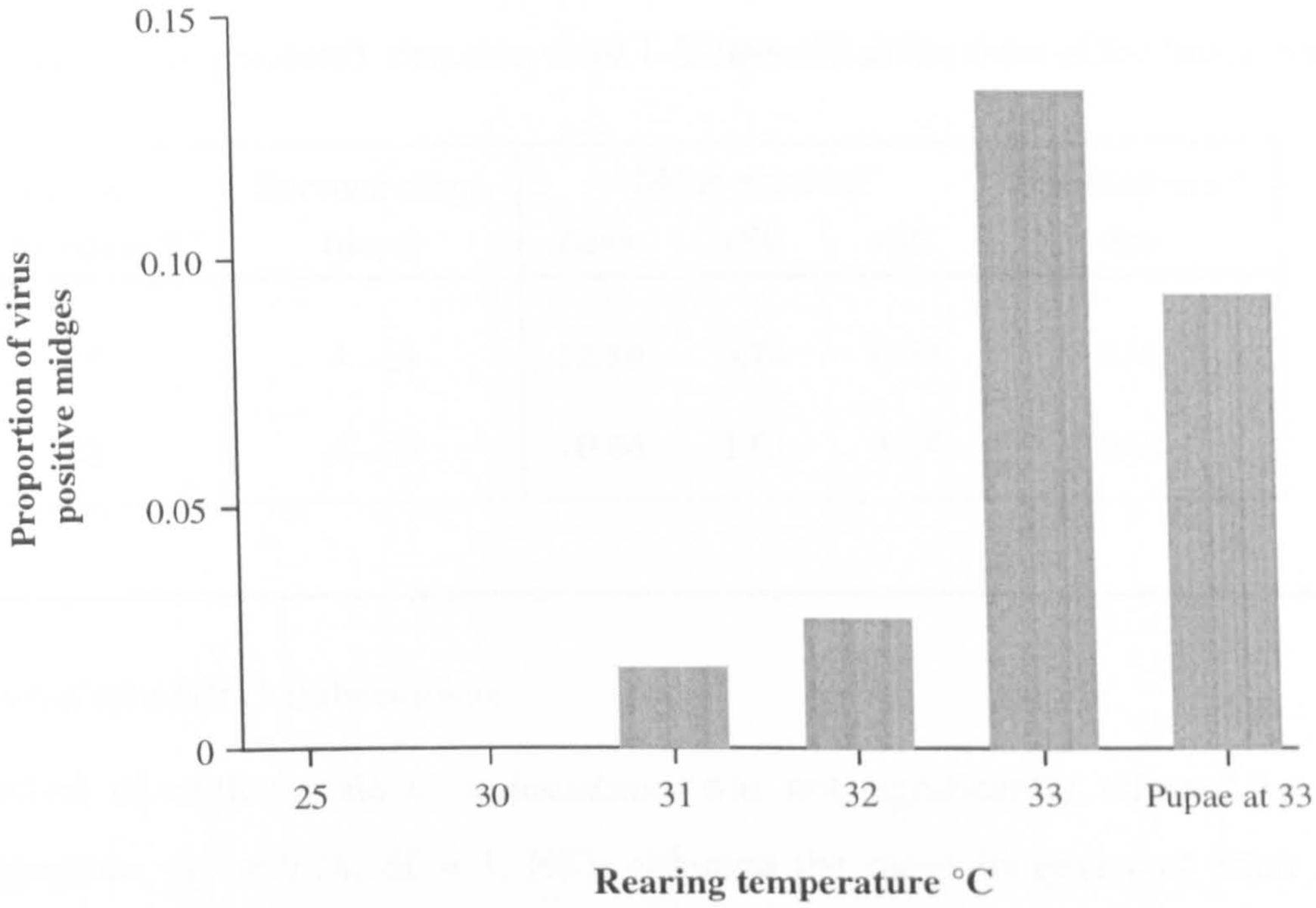
A significantly greater proportion of female *C. nubeculosus* tested positive for the viruses (i.e. viral titre $\geq$1.367 $\log_{10}$ $TCID_{50}$/midge) after being reared for either the whole life cycle or just the pupal stage at 33°C, than at temperatures of 25-32°C (χ^2 = 54.66, df = 1, $p < 0.001$; Figure 5.1). In addition, virus species affected the oral infection rate and a greater proportion of females tested positive for BTV10 compared to AHSV4 (χ^2 = 8.48, df = 1, $p < 0.005$; Figure 5.1). The interaction between rearing temperature and virus species was not significant (χ^2 = 0.02, df = 1, NS), indicating that the impact of rearing temperature on the oral infection rate of female *C. nubeculosus* was similar for BTV10 and AHSV4 (Figure 5.1).

Viral titres in positive midges ranged from 1.867 to 4.2 $\log_{10}$ $TCID_{50}$/midge. Viral titres of BTV10 and AHSV4 obtained in the positive midges reared at 33°C were not significantly different ($F_{1,44}$ = 0.75, NS; Table 5.1). In addition, the length of exposure to 33°C (i.e. for the whole life cycle or just the pupal stage) did not significantly affect the titre of virus obtained in the positive midges ($F_{1,43}$ = 0.09, NS; Table 5.1). The interaction between virus species and length of exposure to 33°C during immature development was also not significant ($F_{1,42}$ = 0.08, NS; Table 5.1).

Table 5.1 Geometric mean viral titres in positive *C. nubeculosus*, reared at 33°C and maintained as adults at 25°C for 10 days after a virus-infected blood-meal.

33°C	Mean titre/positive midge ± SE ($\log_{10}$ $TCID_{50}$/midge)	
	BTV10	**AHSV4**
Whole life cycle	2.92 ± 0.13	2.74 ± 0.18
Pupal stage	2.94 ± 0.13	2.87 ± 0.20

a. BTV10



b. AHSV4

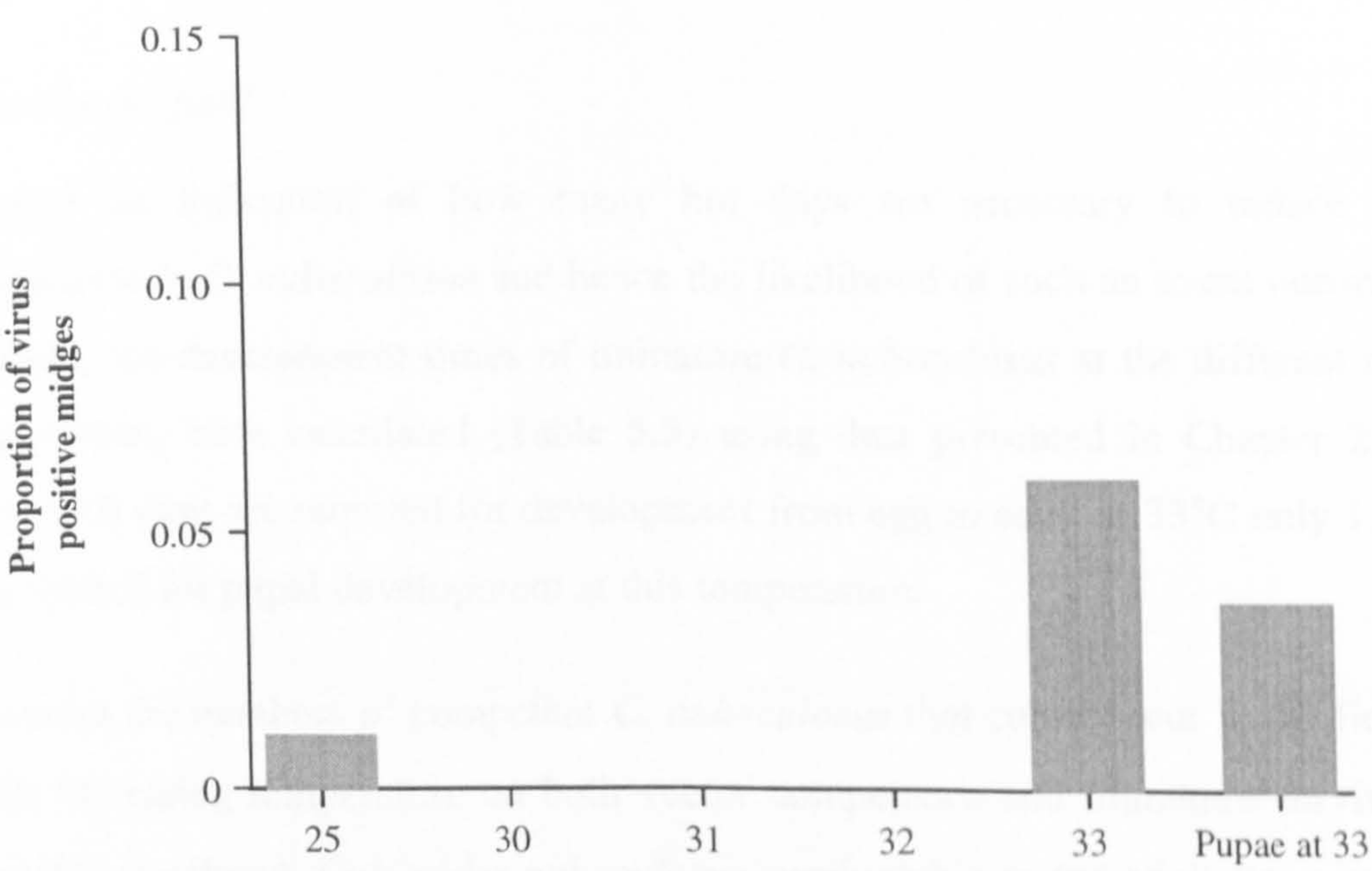


Figure 5.1 Effect of rearing temperature on the proportion of female *C. nubeculosus* that tested positive (i.e. viral titre ≥1.367 $\log_{10}$ $TCID_{50}$/midge) for (a) BTV10 and (b) AHSV4, following the virus infected blood-meal and 10 days incubation at 25°C. One hundred *C. nubeculosus* were tested at each rearing temperature.

Table 5.2 Survival of blood–fed female *C. nubeculosus* reared at 25 and 33°C and maintained as adults at 25°C and 75% relative humidity (based on 50 females from each rearing temperature). Females were 1-2 days old at the time of the blood-meal.

Rearing temperature °C	Survival range (days)	Mean survival days	+SE	-SE	Survival rate / day
25	4 - 21	12.59	0.74	0.67	0.92
33	4 - 17	10.66	1.61	1.24	0.91

Survival of adult C. nubeculosus

Survival of adult female *C. nubeculosus* was not significantly affected by rearing temperature (χ^2 = 0.74, df = 1, NS), although the mean longevity of adult midges reared at 33°C was almost two days shorter than that for adults reared at 25°C (Table 5.2).

Impact in the field

To give an indication of how many hot days are necessary to induce vector competence in *C. nubeculosus* and hence the likelihood of such an event occurring in the field, the development times of immature *C. nubeculosus* at the different rearing temperatures were calculated (Table 5.3) using data presented in Chapter 2. Thus while 16.8 days are required for development from egg to adult at 33°C only 1.8 days are required for pupal development at this temperature.

To assess the numbers of competent *C. nubeculosus* that could occur in the field, the effect of rearing temperature on both vector competence and immature survivorship must be considered. *Culicoides nubeculosus* survivorship to the adult stage increases linearly between rearing temperatures of 12.5 and 30°C but declines rapidly between 31 and 35°C, as the upper lethal rearing temperature of 35°C is approached (Chapter 2). Hence a situation arises whereby few individuals will survive to adulthood at temperatures close to the upper lethal limit but those that do will have an increased chance of being vector competent, while at lower temperatures lots of individuals will survive but few will be capable of transmitting virus.

Rearing temperature °C	Development time of *C. nubeculosus* (days)	No. *C. nubeculosus* surviving to adulthood / 100 eggs	No. female *C. nubeculosus* surviving to adulthood / 100 eggs	BTV10		AHSV4	
				Proportion of positive females	No. competent female *C. nubeculosus*	Proportion of positive females	No. competent female *C. nubeculosus*
25	24.7	53	27	0	0	0.0102	0
30	19.0	64	32	0	0	0	0
31	18.2	49	25	0.0164	0	0	0
32	17.5	35	18	0.0263	0	0	0
33	16.8	23	12	0.1338	2	0.0612	1
Pupae at 33	1.8	46	23	0.0917	2	0.0364	1

Table 5.3 Estimates of the development times of *C. nubeculosus* and of the number of immatures that would survive to adulthood and be susceptible to BTV10 or AHSV4 from an initial starting sample of 100 eggs, at different rearing temperatures.

To investigate this, a situation in which a female *C. nubeculosus* lays a batch of 100 eggs was considered. Initially, the number of eggs that survive to adulthood at the different rearing temperatures was determined using data presented in Chapter 2. For midges exposed to 33°C for just the pupal stage, the number of midges that survive to the pupal stage at 25°C was combined with pupal survival at 33°C. Since the sex ratio of *Culicoides* tends to be 1:1, half of the individuals that survive to adulthood are likely to be female. The number of these females which would be susceptible to BTV10 and AHSV4 was then calculated (Table 5.3). Thus potentially 2 BTV10 or 1 AHSV4 competent *C. nubeculosus* female could develop for every 100 individuals reared at 33°C either for the whole life cycle or just the pupal stage. In contrast, all individuals reared at 25-32°C are unlikely to be capable of transmitting BTV10 and AHSV4.

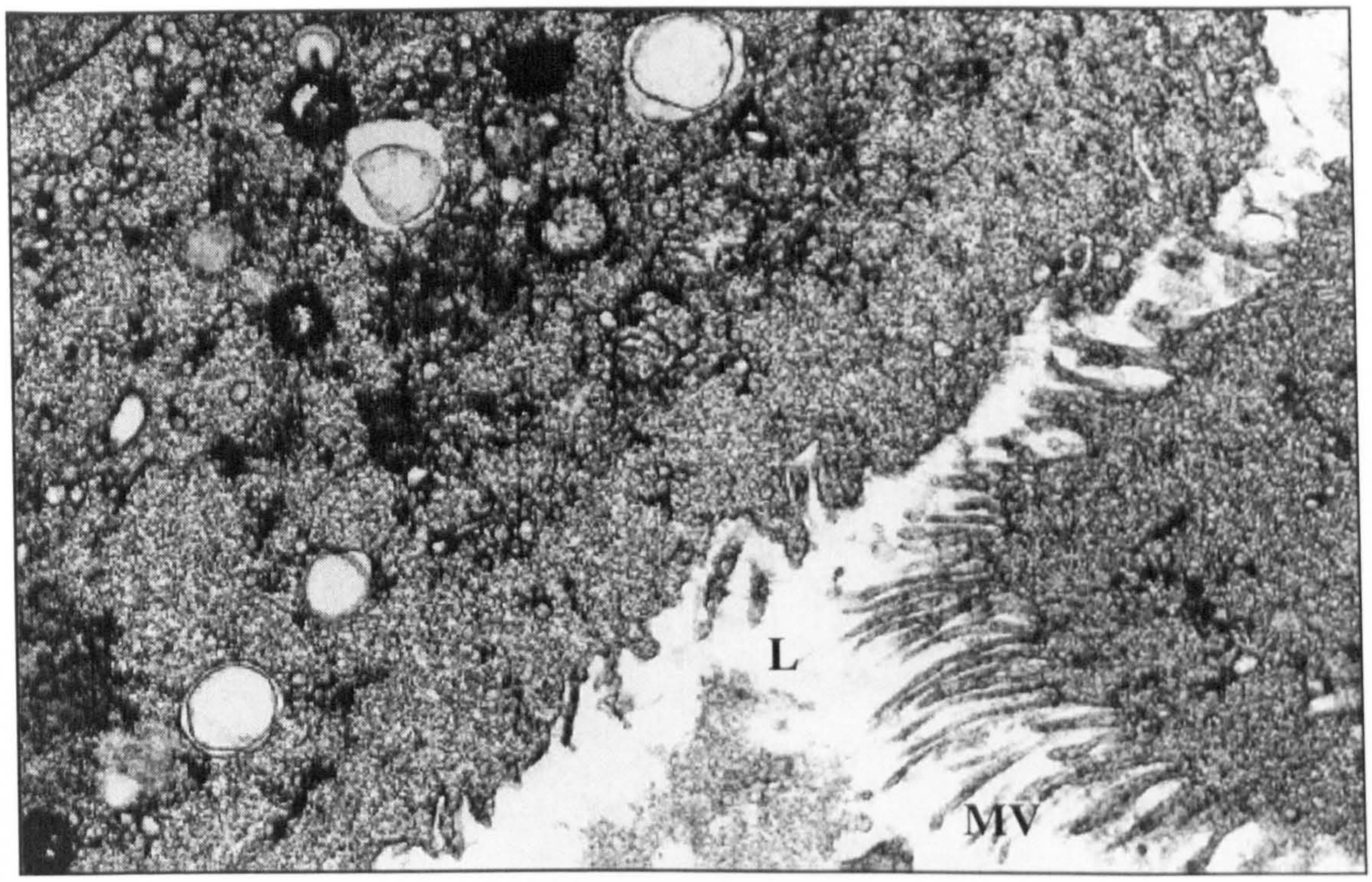
Electron microscopy

Although the interpretation of the electron micrographs is speculative, there appear to be differences between the guts of *C. nubeculosus* reared at control and elevated temperatures. For example, microvilli on the luminal surface of the gut cells in midges reared at 33°C appear disorganised and have lost their rounded turgid appearance (Figure 5.2). The basement membrane lining the gut cells of midges reared at 33°C appears to be thinner than in midges reared at 25°C (Figures 5.3b and 5.4). Furthermore, in midges reared at 33°C one hour after a blood-meal, the same granular material appears on both sides of the gut cells, as though there was leakage of material from the gut lumen to the exterior (Figure 5.4).

5.5 Discussion

Rearing temperatures close to the upper lethal limit significantly increased the oral infection rate of *C. nubeculosus* for both BTV10 and AHSV4. Thus 13.4% and 6.1% of females that were reared at 33°C tested positive for BTV10 and AHSV4 respectively, compared to <3% of females that tested positive at rearing temperatures of 25 to 32°C. Due to the impact of rearing temperature on immature survivorship and vector competence, potentially two BTV10 and one AHSV4 competent female could develop for every batch of 100 eggs reared at 33°C. Exposure to elevated temperatures during the immature stages did not affect the survival of adult *C. nubeculosus*.

a.



b.

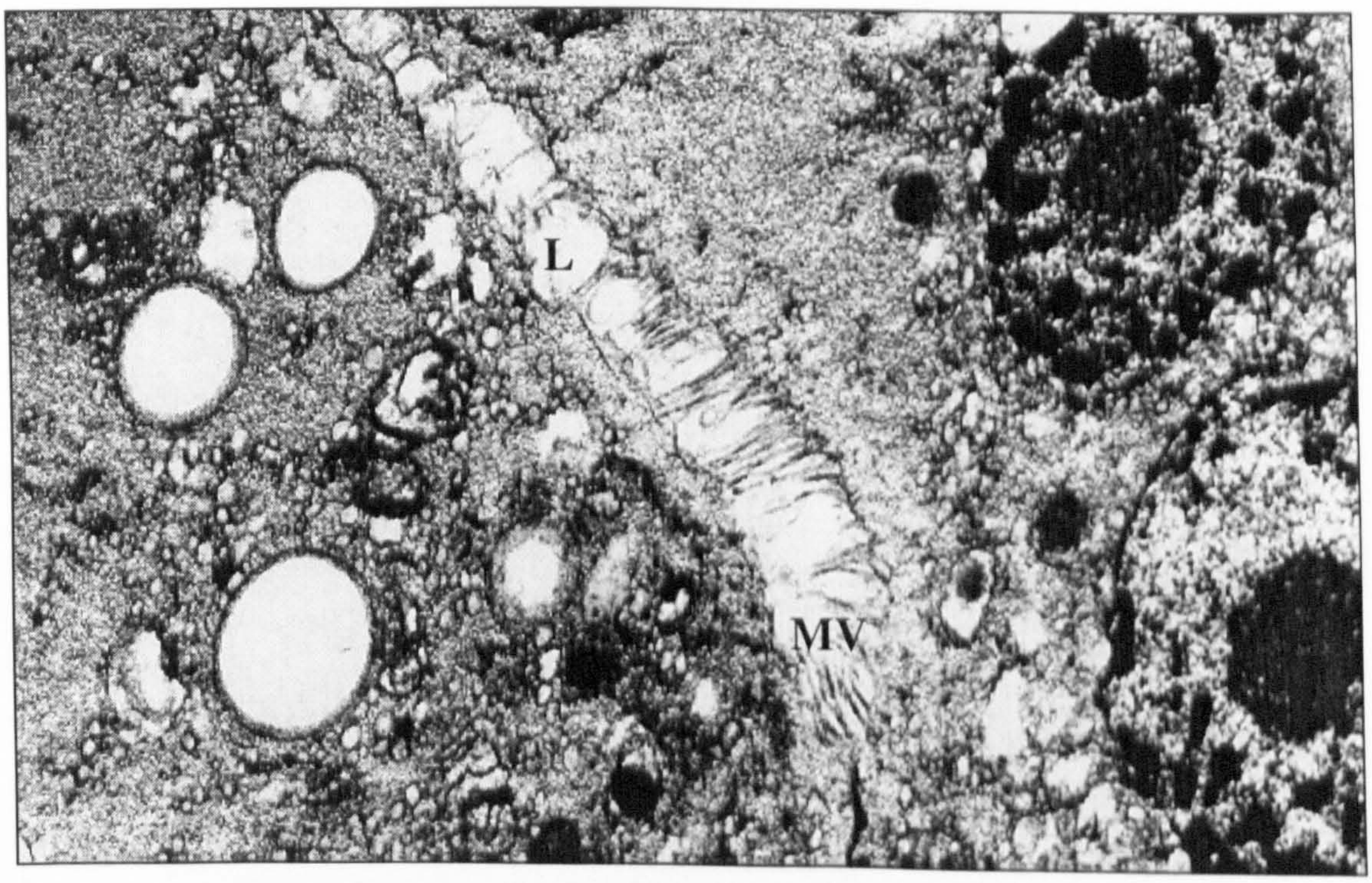
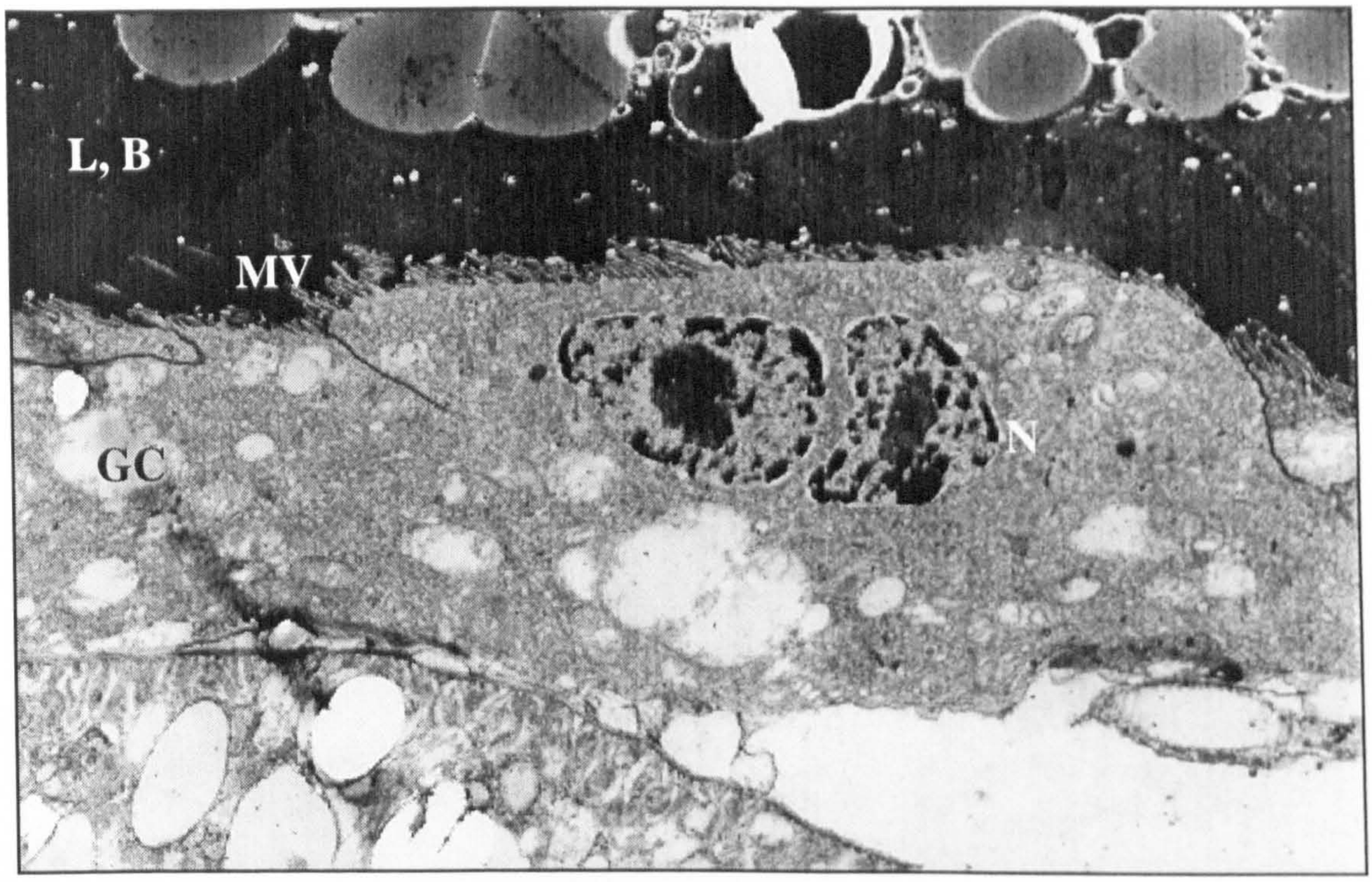


Figure 5.2 Electron micrographs of the midgut of *C. nubeculosus* reared at (a) 25°C and (b) 33°C and which had not been blood-fed. Original magnification x 18000. MV = microvilli and L = gut lumen.

a.



b.

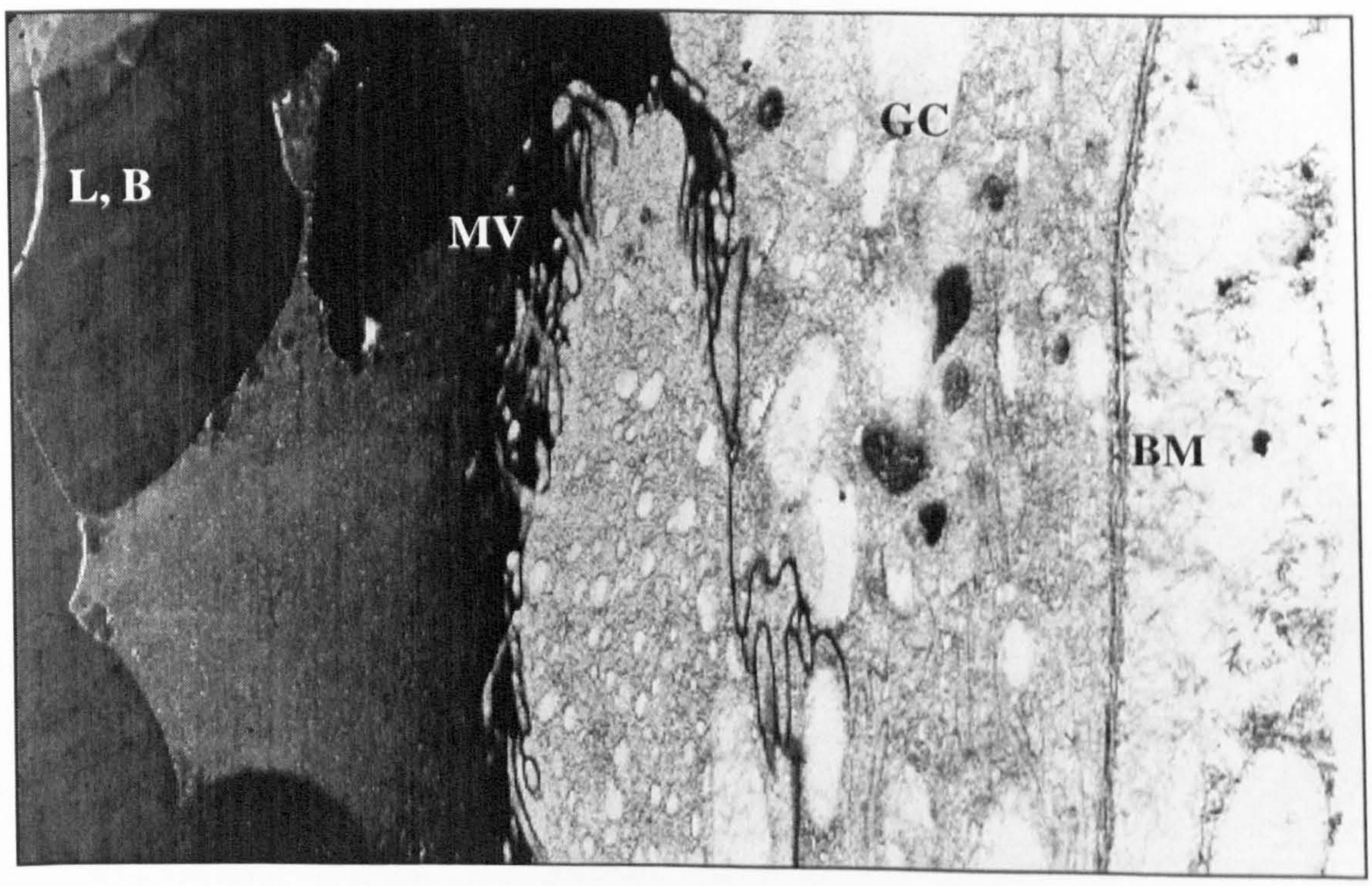
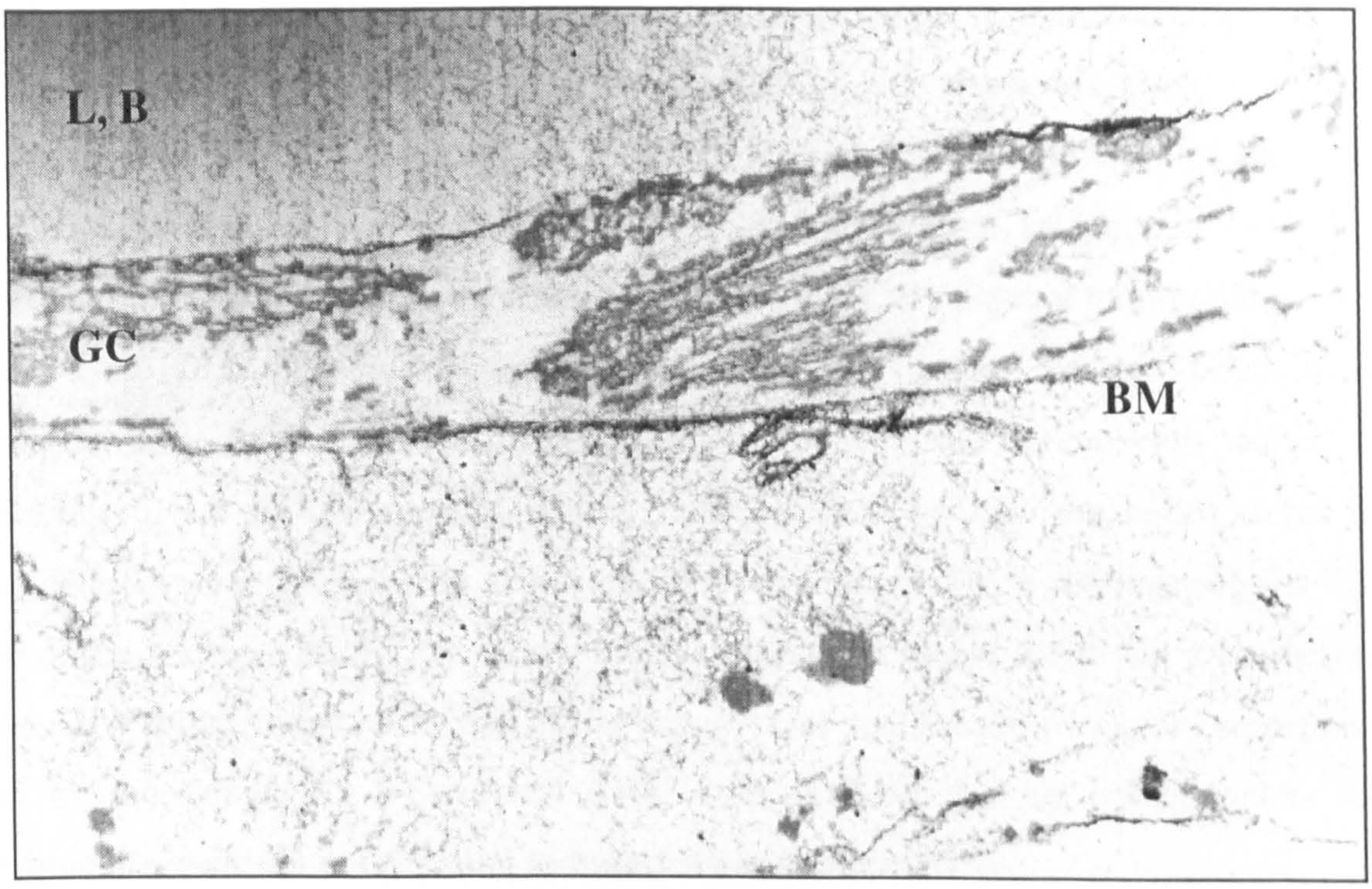


Figure 5.3 Electron micrographs of the midgut of *C. nubeculosus* reared at 25°C, 1 hour after a blood-meal. Original magnification (a) x 6500 and (b) x 18000. L = gut lumen, B = blood, MV = microvilli, N = nucleus, GC = gut cells and BM = basement membrane.

a.



b.

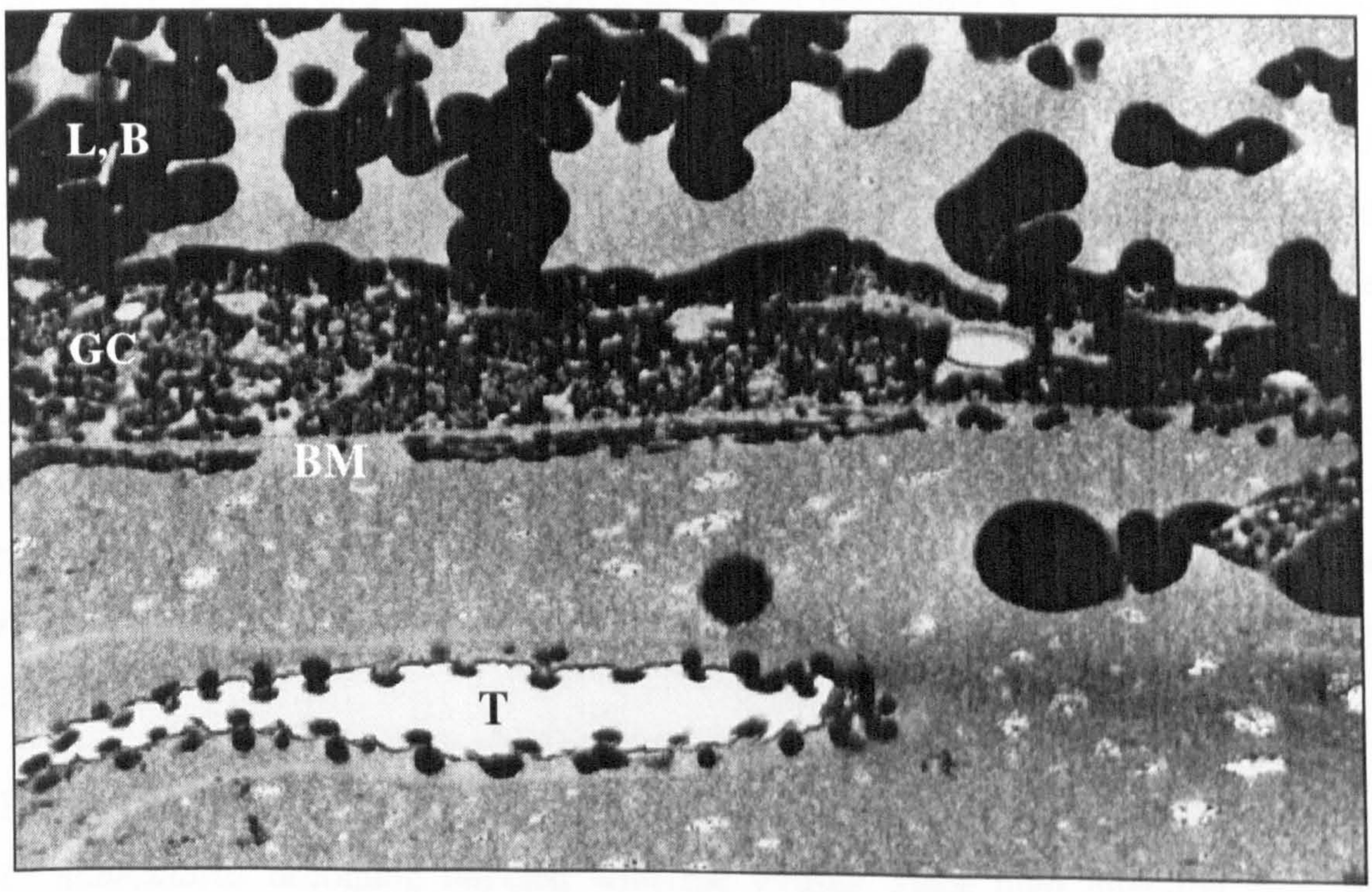


Figure 5.4 Electron micrographs of the midgut of *C. nubeculosus* reared at 33°C, 1 hour after a blood-meal. Original magnification x 18000 for both (a) and (b). L = gut lumen, B = blood, GC = gut cells, BM = basement membrane and T = trachea. The same granular material occurs on both sides of the gut epithelium.

Exposure of pupae to 33°C was also sufficient to induce vector competence in *C. nubeculosus* for both viruses. However, although the oral infection rates of *C. nubeculosus* reared at 33°C for the pupal stage or the whole life cycle were not significantly different, lower infection rates with both BTV10 and AHSV4 occurred when only the pupae were exposed to 33°C. In the Lepidoptera, midgut epithelial cells present in the adults are formed a short time before the larval/pupal moult (Baldwin *et al.*, 1996). If *C. nubeculosus* development is similar, then by the time pupae were transferred to 33°C, the adult midgut epithelial cells would have already started to develop. Thus the lower oral infection rates could be because the initial stages of midgut development were not subjected to hot conditions. These findings suggest that high temperatures during the development of the adult midgut cells (i.e. from before the larval/pupal moult to the end of the pupal stage) could result in vector competence of *C. nubeculosus* for BTV10 and AHSV4 and indicate that hot conditions for the whole life cycle are not essential to induce vector competence.

The higher oral infection rate of *C. nubeculosus* with BTV10 compared to AHSV4 could have been because horse blood was used as the source of blood for the blood-meals. Sieburth *et al.* (1991) showed that when a vector species, *C. variipennis sonorensis*, ingested a blood-meal of sheep blood and BTV, some of the BTV particles bound to the red blood cells (RBCs), while the free virus particles infected the midgut cells. In the case of AHSV4 with horse blood, it is possible that a large proportion of the virus particles bind to the horse RBCs, leaving a small proportion of free virus particles to initiate infection. However, the affinity of BTV10 particles to the horse blood cells may be low, as horses are not a host for BTV. A larger proportion of free virus particles may therefore have been available to initiate infection, thereby accounting for the higher oral infection rate of *C. nubeculosus* with BTV10.

Horse blood was used for the virus infected blood-meals to maximise the likelihood of *C. nubeculosus* becoming infected with the viruses, since high infection rates for AHSV (Marchi *et al.*, 1995) and BTV (Burroughs, unpub. obs.) in *C. variipennis sonorensis* were obtained when horse blood was used. Although the susceptibility of a vector species to orbiviruses can vary with the virus species and serotype (Jones and Foster, 1978; Bellis *et al.*, 1994; Mecham and Nunamaker, 1994; Venter *et al.*, 1998;

Chapter 3), the fact that higher oral infection rates in *C. nubeculosus* were obtained with BTV10, where there may have been lots of free virus in the blood-meal, compared to AHSV4, where many virus particles may have bound to RBCs, suggests that *C. nubeculosus* becomes infected with the viruses via a different route to that which would occur in a genetically competent species.

For example, if vector competence in *C. nubeculosus* is due to gut damage, it is likely that viral infection will occur within a limited time of ingesting the blood-meal. This is because the damage is likely to be accentuated when the gut is distended with blood, increasing the likelihood of virus particles entering the haemocoel. In addition, the peritrophic membrane of *C. nubeculosus*, which forms around the blood-meal, develops by five hours after ingestion (Megahed, 1956). Once the virus particles are enclosed by the peritrophic membrane, they may be unable to initiate infection until the membrane begins to break down ≈2-3 days later. However by this time the gut will no longer be distended. Hence a high concentration of free virus particles present in the gut shortly after a blood-meal will increase the chance of infection. In contrast, with genetically competent individuals there is no such time restriction on when virus particles can infect the midgut cells and the association of virus particles with RBCs therefore does not hinder the infection process. Thus when the blood cells are digested, up to 3 days after the blood-meal, the bound virus particles are released and can subsequently infect the midgut cells.

The oral infection rate of *C. nubeculosus* with BTV10, when the immatures were reared at 33°C, was therefore probably higher than would have occurred if a more natural system been employed (e.g. BTV and sheep blood). The lower oral infection rate of *C. nubeculosus* with AHSV4, may provide a better indicator of what would have happened in a natural system. However, the fact that an increased oral infection rate was still obtained with AHSV4 and high titres were present in the positive midges, suggests that vector competence due to elevated rearing temperatures is a real phenomenon.

Preliminary electron microscopy trials revealed that there were differences in the midgut tissue of *C. nubeculosus* reared at control and elevated temperatures. However, further trials using greater numbers of midges are necessary to establish the

exact nature of these differences. For example, although elevated rearing temperatures appeared to result in the thinning of the basement membrane, measurements must be taken to confirm this. Grimstad and Walker (1991) found that *Aedes triseriatus* with thinner basement membranes were more likely to develop disseminated La Crosse virus infections and suggested that the thinner basement membrane presented a less obstructed passage for the virus into the haemocoel. Hence it is possible that the thickness of the basement membrane could affect the ability of *C. nubeculosus* to transmit BTV and AHSV. It is also necessary to confirm whether material can pass from the gut lumen to the haemocoel by an intercellular pathway following a blood-meal in midges reared at elevated temperatures and to establish whether there are any further differences in the gut tissue. In addition, it would be interesting to investigate whether the ovariole sheath (i.e. outer covering of the ovariole) is affected by rearing temperature, since it also consists of a layer of epithelial cells resting on a basement membrane. Fu (1996) found that the ovariole sheath of *Culicoides* acts as a barrier preventing virus from entering the developing eggs. Hence if rearing temperature does affect the ovariole sheath, it is possible that it may also influence the ability of *C. nubeculosus* to transovarially transmit BTV and AHSV.

If vector competence for BTV10 and AHSV4 in adult *C. nubeculosus* reared at high temperatures is indeed due to gut damage, then it is likely that they will also be susceptible to the other BTV and AHSV serotypes. In addition, since most arboviruses replicate in the haemocoel when the midgut barriers are bypassed, hot temperatures may not only induce vector competence for orbiviruses but also for a wide variety of other arboviruses. Thus under suitable conditions *C. nubeculosus* could become a 'universal vector' (Mitchell, 1983). In addition, the effects of high temperatures on vector competence may not be restricted to *C. nubeculosus* but also occur in other 'non-vector' *Culicoides* species. For example, Mellor *et al.* (1998) speculated that field isolations of AHSV4 made from mixed pools of *C. obsoletus* and *C. pulicaris* in Spain (Mellor *et al.*, 1990) could be the result of the 'leaky gut' phenomenon.

However, the requirement of such high temperatures (i.e. 33°C) to induce vector competence in *C. nubeculosus* will restrict the prevalence of this mechanism both spatially and temporally. Nevertheless, the fact that hot conditions for the whole life cycle are not essential and that just two days of hot conditions during the pupal stage

can induce competence, increases the chances of susceptible *C. nubeculosus* occurring in the field. The increase in frequency and intensity of extremely warm days predicted to occur with climate change (Houghton, 1997) will also enhance the chances of such an event occurring.

Thus despite the need for relatively hot temperatures, it is possible that competent *C. nubeculosus* could occur in the field. The implications of this are particularly worrying with regards to the risk of BTV and AHSV in Europe. Lord *et al.* (1996) have shown that vector numbers are critical in determining both the likelihood and severity of an outbreak, following the introduction of virus. Hence epidemics of BTV or AHSV in areas where *C. imicola* (the principal European vector) and *C. nubeculosus* coexist could become more severe, if hot conditions resulted in *C. nubeculosus* also being able to transmit virus. In addition, *C. nubeculosus* is much more widely distributed in Europe than *C. imicola.* Hence under the right conditions it is possible that competent *Culicoides* could occur in areas not previously considered to have competent *Culicoides* populations. For example, temperatures as high as 33.9°C have been recorded in London during July (Anon, 1996), so that even parts of the UK could be at risk of BTV and AHSV.

CHAPTER 6: USING CLIMATE DATA TO MAP THE POTENTIAL DISTRIBUTION OF *CULICOIDES IMICOLA* (DIPTERA: CERATOPOGONIDAE) IN EUROPE.

6.1 ABSTRACT

Culicoides imicola, a vector of bluetongue virus and African horse sickness virus, is principally Afro-Asian in distribution, but has recently been found in parts of Europe, including Iberia and Greece. A logistic regression model based on climate data (temperature, saturation deficit, rainfall and altitude) and the published distribution of *C. imicola* in Iberia was developed and then applied to other countries in Europe, to identify where *C. imicola* could become established. The model identified three temperature variables (minimum of the monthly minimum temperature, maximum of the monthly maximum temperature and number of months with mean temperature ≥12.5°C) as significant determinants of the distribution of *C. imicola* in Iberia and indicated that under current conditions *C. imicola* could extend its distribution in Spain and Greece. Although areas of Italy and Albania were also identified as suitable for *C. imicola*, its presence in these regions is initially dependent on its spread from eastern to western Greece, which is likely to be hindered by local topography. To simulate the effect of global warming, temperature values in the model were increased by 2°C. Under these conditions, the potential spread of *C. imicola* in Spain and Greece will be even more extensive than that predicted under current conditions.

6.2 INTRODUCTION

The biting midge *Culicoides imicola* (Diptera: Ceratopogonidae) is found in sub-Saharan Africa (Meiswinkel, 1989), North Africa (Macfie, 1943; Szadziewski, 1984; Baylis *et al.*, 1997) and southern Asia, as far east as Laos (Howarth, 1985) and Vietnam (Wirth and Hubert, 1989). It is also found in parts of Europe, including south-western Iberia (Rawlings *et al.*, 1997) and the Greek islands of Lesbos (Boorman and Wilkinson, 1983), Rhodes (Boorman, 1986), Chios, Kos, Samos (Mellor, pers. comm.) and Evia (Patakakkis, unpub. obs.). In addition, it was recently discovered on mainland Greece, in the provinces of Chalkidhiki, Larisa and Magnisia (Patakakkis, unpub. obs.) and it is likely that it will expand its range into further areas of Europe (e.g. Rawlings *et al.*, 1998).

Table 6.1 Outbreaks of bluetongue and African horse sickness in Europe.

Outbreak	Reference
Bluetongue	
Portugal and Spain 1956-60	Campano Lopez and Sanchez Botija (1958)
Lesbos 1979	Vassalos (1980)
Rhodes 1980	Dragonas (1981)
Mainland Greece, Chios, Evia, Kos, Lesbos, Rhodes and Samos 1999	Anon (1998), Anon (1999d,e), Anon (2000b)
Bulgaria 1999	Anon (1999b)
European Turkey 1999	Anon (1999c)
African horse sickness	
Spain 1966	Diaz Montilla and Panos Marti (1968)
Spain and Portugal 1987-1990	Rodriguez *et al.* (1992)

Culicoides imicola is a vector of several arboviruses of domestic and wild animals, including bluetongue virus (BTV), which infects ruminants and African horse sickness virus (AHSV), which infects equids. The diseases caused by these viruses, bluetongue (BT) and African horse sickness (AHS), are of such major international concern that they have attained OIE list 'A' status. Although BT and AHS are not endemic in Europe, several outbreaks have occurred in the south (Table 6.1). *Culicoides imicola* has been implicated as the principal vector species in these outbreaks (Mellor, 1990; Ortega *et al.*, 1998; Patakkakis, unpub. obs.), except for the BT outbreak in Bulgaria, where *C. imicola* does not seem to occur (Mellor, pers. comm.).

Areas where *C. imicola* is found are potentially at risk of BT and AHS. Consequently, it is useful to identify where *C. imicola* could become established in Europe. Climate is a major factor governing the distribution of *C. imicola*. For example, there is some evidence that the northern limit of *C. imicola* in Iberia is determined by low temperature (Baylis and Rawlings, 1998). Where temperatures are favourable,

precipitation may influence distribution through its impact on the availability of breeding sites. *Culicoides imicola* breeds in wet, organically enriched soil or mud (Walker and Davies, 1971; Braverman *et al.*, 1974; Lubega and Khamala, 1976; Walker, 1977; Braverman, 1978) and in Africa it tends to occur in regions with an annual rainfall of 300-700 mm (Meiswinkel and Baylis, 1998). One approach to establish where *C. imicola* could occur is therefore to identify the most significant climatic determinants of its current distribution in Europe and then from this, derive 'expected' distributions for other parts of Europe.

The present study was therefore carried out to identify the most important climatic factors influencing the distribution of *C. imicola*, using published data on the presence and absence of *C. imicola* in Iberia (Rawlings *et al.*, 1997) together with climate data from this region (Anon, 1972). The derived climatic model can then be used to identify other areas of Europe which have suitable climates for the occurrence of *C. imicola*. In addition, with a 2°C rise in the global mean temperature expected to occur during the next 100 years (Intergovernmental Panel on Climate Change, 1996), it is likely that *C. imicola* could extend its range even further in Europe (e.g. 2°C change in the mean annual temperature corresponds to a northward shift of ≈200 km; Hughes, 2000). This increase in temperature can then be incorporated into the model, to investigate how climate change may affect the distributional range of *C. imicola* in Europe.

6.3 METHODS

Climate data for sites in Iberia were obtained from Anon (1972) and were based on the average values for the period 1931-60. Thirty sites occurred within regions for which there is information about the occurrence of *C. imicola* (Rawlings *et al.*, 1997). *Culicoides imicola* was classed as present at 16 sites and absent from 14 sites (Figure 6.1).

Climatic Variables

Temperature variables for the 30 sites used in the analysis included minimum of the monthly minimum, minimum of the monthly mean, mean of the monthly mean, maximum of the monthly mean, maximum of the monthly maximum, number of months with mean temperatures ≥10.5°C, number of months with maximum temperatures

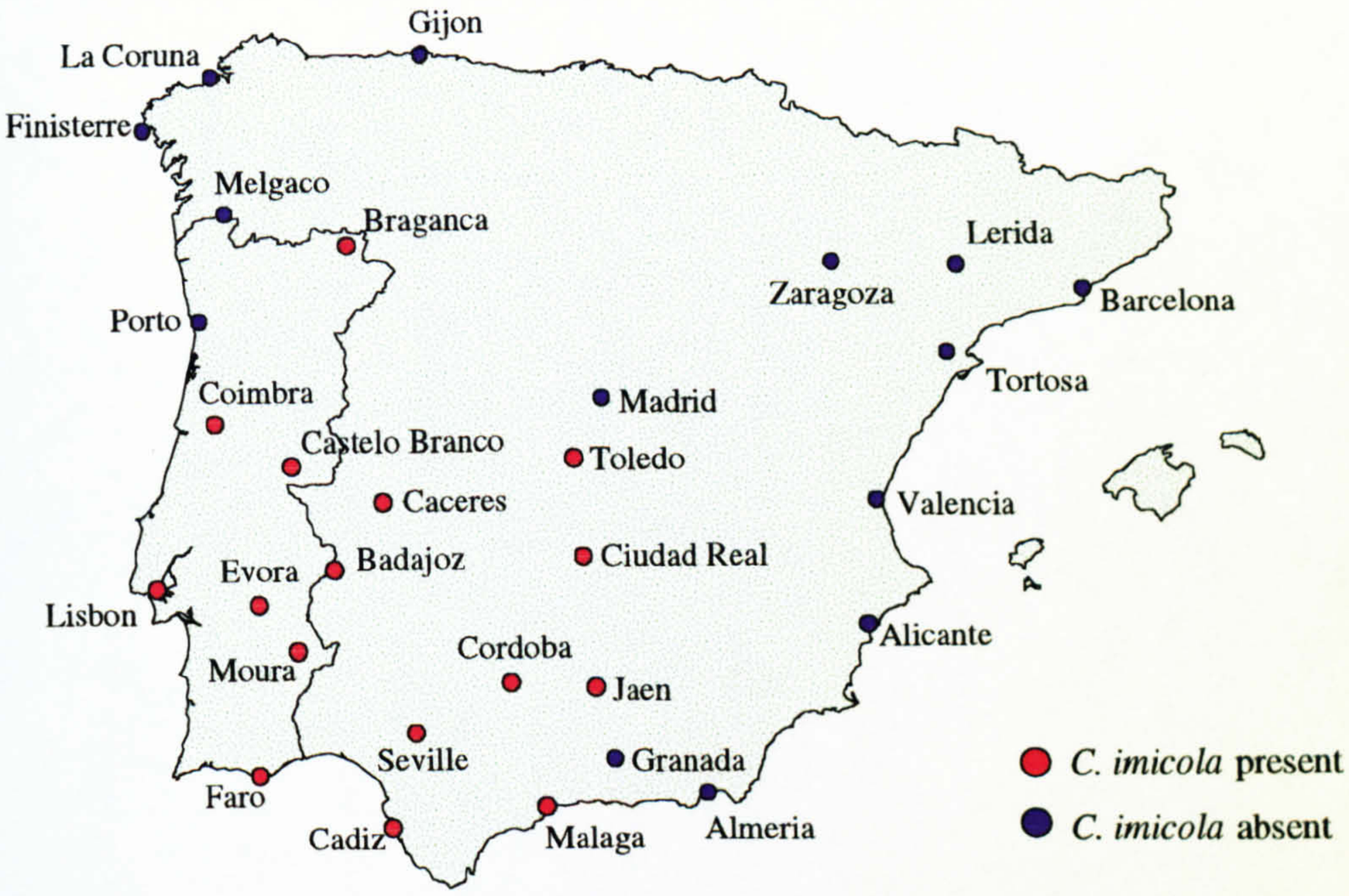


Figure 6.1 Sites included in the logistic regression analysis of the distribution of *C. imicola* in Iberia. The presence or absence of *C. imicola* at the sites was determined from Rawlings *et al.* (1997).

≥12.5°C and number of months with mean temperatures ≥12.5°C. The number of months with mean temperatures ≥10.5°C was included, since this is the lowest temperature at which successful immature development to adulthood can occur in the model species, *C. variipennis sonorensis* (Chapter 2). The number of months with maximum temperatures ≥12.5°C was incorporated as Sellers and Mellor (1993) found that *C. imicola* adults could only survive the winter in areas where the average daily maximum temperature during the coldest month of the year (i.e. minimum of the monthly maximum temperature) was ≥12.5°C. However, while adults may survive when the maximum temperature during the coldest month is ≥12.5°C, their activity is likely to be limited. Consequently, the number of months with mean temperatures ≥12.5°C was also considered.

The annual daily mean saturation deficit (Appendix 2), a measure of the drying power of air based on both air temperature and relative humidity, was also included for each site. This was determined by averaging the annual daily maximum and minimum deficits: the former was calculated from the annual average daily maximum temperature and annual average daily minimum relative humidity (highest saturation deficit); the latter from the annual average daily minimum temperature and the annual average daily maximum relative humidity (lowest saturation deficit).

The total annual rainfall and altitude for each site were also included in the analysis.

Analysis

Logistic regression, carried out in Glim 3.77, was used to determine which climatic variables were most important in distinguishing between sites where *C. imicola* is present and absent. The derived model was then used to calculate the probability of occurrence of *C. imicola* at each of the 30 sites. *Culicoides imicola* was classed as present at sites with a probability of occurrence value of ≥0.5 and absent from sites with a value <0.5. The predicted presence or absence of *C. imicola* at each site was compared with the published data (Rawlings *et al.* 1997; Figure 6.1), in order to assess the reliability of the model. The model was then applied to additional sites in Iberia for which there is no information about the occurrence of *C. imicola* and was also used to assess the suitability of sites in Albania, Bosnia, Bulgaria, Croatia, European Turkey, France, Greece, Italy, Macedonia, Slovenia, Switzerland and Yugoslavia. To simulate climate change the temperature values were increased by 2°C and the model was then reapplied to the sites in Europe.

6.4 RESULTS

Mean values for the climatic variables at the 30 sites in Iberia used to produce the logistic regression model are shown in Table 6.2. Three climatic variables - minimum of the monthly minimum temperature, maximum of the monthly maximum temperature and the number of months with mean temperature ≥12.5°C were significant in distinguishing between sites where *C. imicola* is present and absent (Table 6.2).

Table 6.2 Mean values (+/-SE) for the climatic variables and results of the logistic regression analysis for the distribution of *C. imicola* in Iberia.

Climatic Variable	Sites with *C. imicola* n = 16	Sites without *C. imicola* n = 14	χ^2 df = 1
Altitude ft	274.4 ± 61.2	184.9 ± 59.1	0.33
Temperature °C			
Minimum of the monthly minimum	5.1 ± 0.7	4.8 ± 0.6	6.39*
Minimum of the monthly mean	8.9 ± 0.6	8.7 ± 0.6	0.35
Mean of the monthly mean	16.5 ± 0.5	15.5± 0.4	2.22
Maximum of the monthly mean	25.0 ± 0.6	23.0 ± 0.7	0.44
Maximum of the monthly maximum	31.8 ± 0.8	28.3 ± 1.0	7.28**
No. months/year mean temperature ≥10.5°C	9.6 ± 0.5	9.4 ± 0.4	0.02
No. months/year maximum temperature ≥12.5°C	11.1 ± 0.3	11.1 ± 0.3	0.04
No. months/year mean temperature ≥12.5°C	8.5 ± 0.4	7.8 ± 0.3	4.34*
Saturation deficit mbar	7.2 ± 0.3	6.0 ± 0.4	0.44
Total annual rainfall mm	607.4 ± 46.4	635.5 ± 86.6	0.71

* $p<0.05$; ** $p<0.01$

The logistic regression model based on these variables is:

$$y = 0.5460*A + 0.6020*B - 0.4243*C - 15.78$$

where A is the minimum of the monthly minimum temperature, B is the maximum of the monthly maximum temperature, C is the number of months with mean temperature ≥12.5°C and y is a logit (i.e. natural log of the odds ratio; ln(p/1-p)). The probability of occurrence of *C. imicola* (p) can then be calculated using the following formula (Crawley, 1993):

$$p = \frac{1}{1+\frac{1}{e^{y}}}$$

The model correctly predicted the presence or absence of *C. imicola* at 25 of the 30 sites (83%). However, *C. imicola* was incorrectly predicted as present at 3 sites (10%) - Granada, Almeria and Alicante in eastern Spain and incorrectly predicted as absent at 2 sites (7%) - Braganca and Coimbra in Portugal.

The suitability of sites in Europe for the occurrence of *C. imicola* based on the model established for Iberia is shown in Figure 6.2. In addition to southern Iberia, parts of southern Italy, Sardinia and Sicily would be suitable for *C. imicola*, should it be introduced into these regions. The majority of sites in Greece would also be favourable, except for parts of northern Greece and the Peloponnese. Several sites along the eastern Adriatic Sea coastline, ranging from Albania to Croatia, would also be suitable. The furthest north that *C. imicola* could potentially occur is Split (43°31'N) in Croatia.

With a 2°C increase in temperature greater areas of Europe will become favourable for *C. imicola* (Figure 6.3). For example, in Spain sites along the east coast will become suitable, although areas along the Atlantic Sea coastline of both Spain and Portugal will remain unfavourable. Sites on the south coast of France and in Corsica will become favourable. The majority of southern Italy extending up the Ligurian Sea coastline e.g. Genoa (44°25'N) will also become suitable. All sites in Greece, except for Florina (40°48'N) near to the border with Macedonia, would become suitable and along the eastern Adriatic Sea coastline *C. imicola* could potentially occur up to Rijeka (45°20'N) in Croatia.

Although annual rainfall was not significant in distinguishing between sites where *C. imicola* is present and absent in Iberia (Table 6.2), it may be an important determinant of the distribution of *C. imicola* in other parts of Europe. Annual rainfall is similar at sites where *C. imicola* is both present and absent in Iberia and is generally within the favourable range of 300-700 mm per year, although *C. imicola* also occurs at some

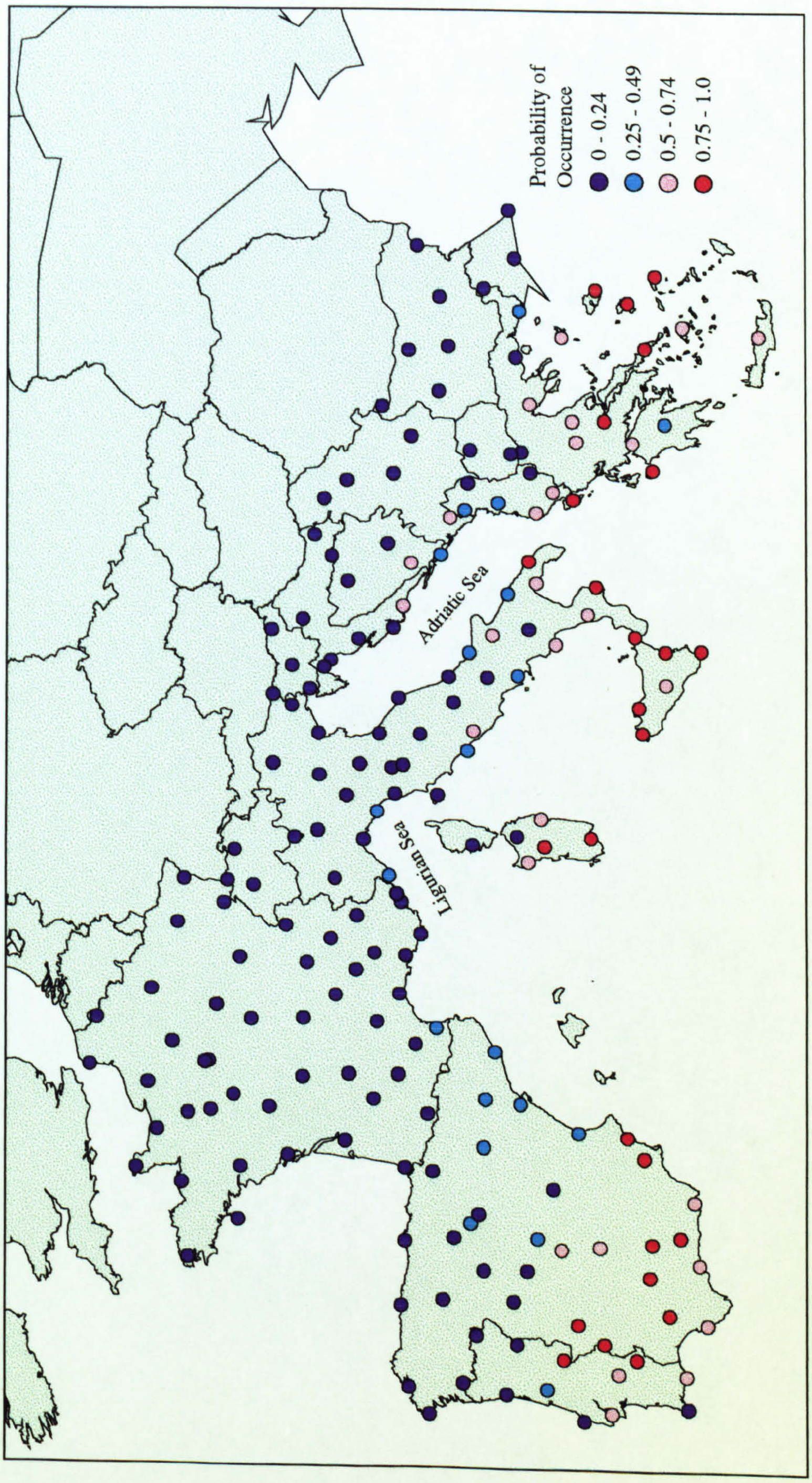


Figure 6.2 Suitability of sites in Europe for the occurrence of *C. imicola* based on the logistic regression model established for Iberia.

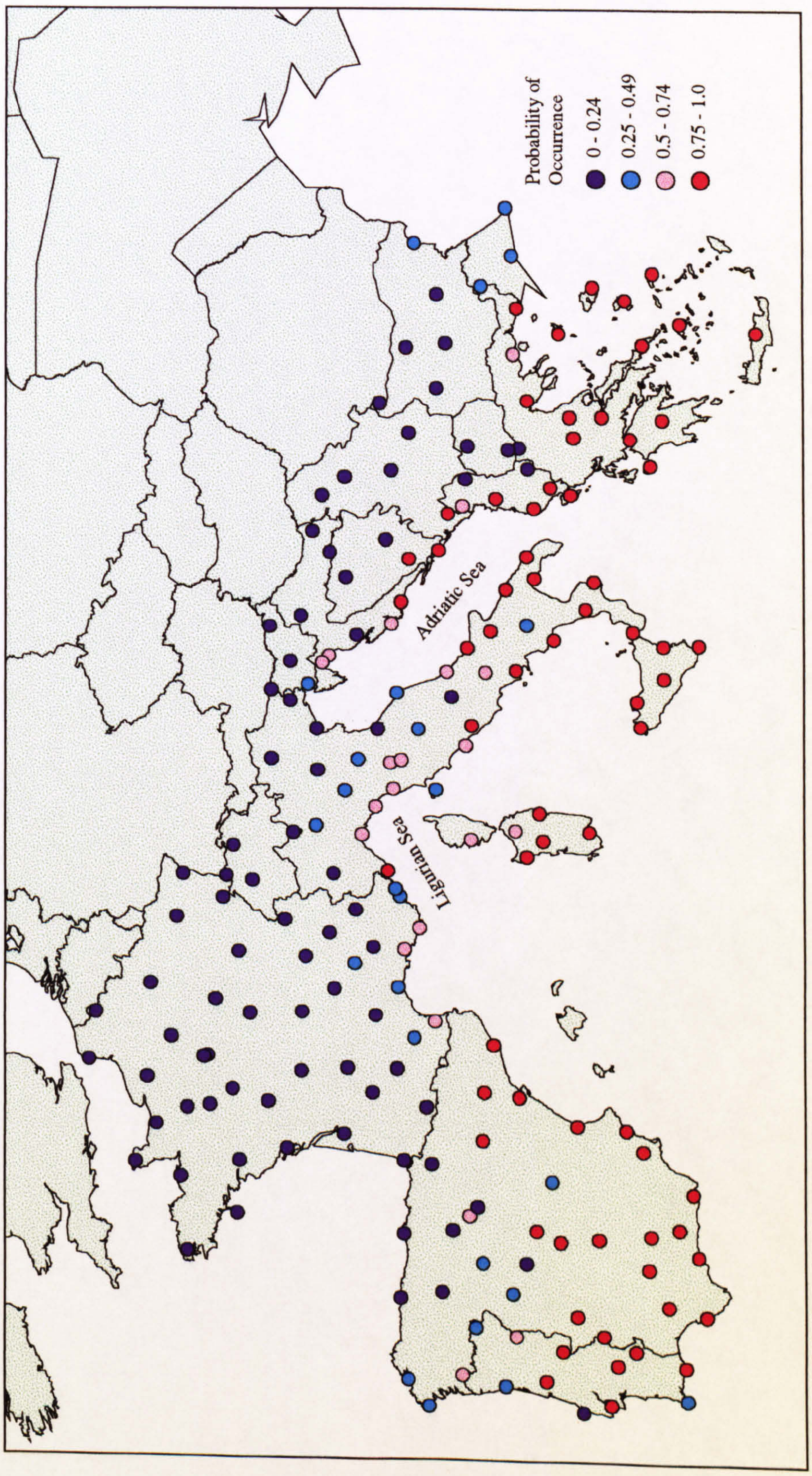


Figure 6.3 Suitability of sites in Europe for the occurrence of *C. imicola* based on the logistic regression model established for Iberia with a 2°C increase in temperature.

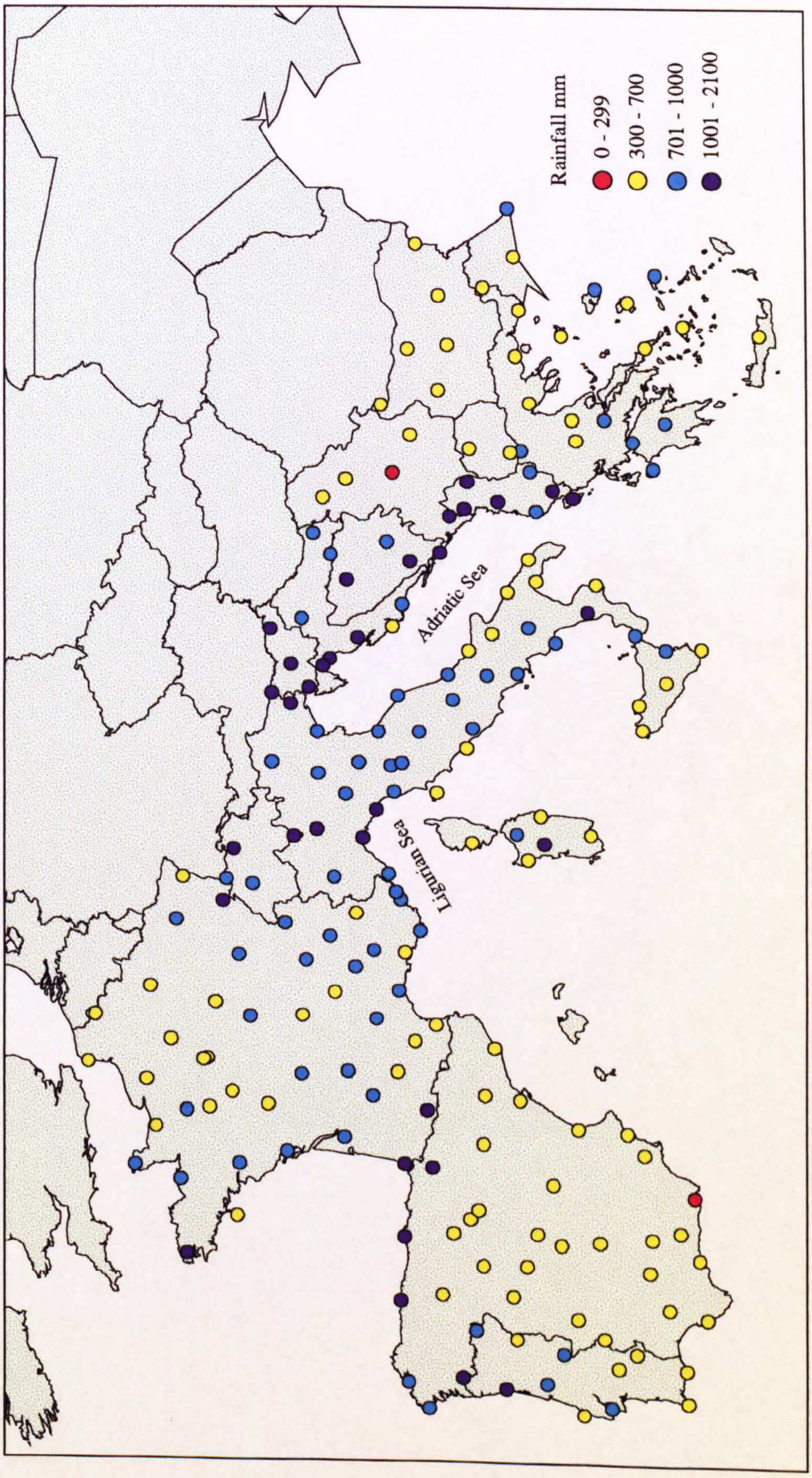


Figure 6.4 Total annual rainfall for sites in Europe.

sites with an annual rainfall of 701-1000 mm (e.g. Braganca, Castelo Branco, Coimbra and Lisbon; Figure 6.4). *Culicoides imicola* pupae drown when breeding sites are flooded (Nevill, 1967) and hence it is likely that sites with an annual rainfall of >1000 mm will be unsuitable for *C. imicola*. Thus while rainfall is not a limiting factor in Iberia, several sites along the eastern Adriatic sea coastline predicted by the model (on the basis of temperature) as suitable for the occurrence of *C. imicola* may in fact be too wet (Figure 6.4). Furthermore, the global mean precipitation is expected to increase as climate changes (Intergovernmental Panel on Climate Change, 1996), which could result in additional sites becoming unsuitable for the occurrence of *C. imicola*.

6.5 Discussion

The distribution of *C. imicola* in Iberia appears to be limited by temperature. The minimum of the monthly minimum temperature, the maximum of the monthly maximum temperature and the number of months with mean temperatures ≥12.5°C were significant determinants of the distribution of *C. imicola*. The model based on these temperature variables displayed a high degree of accuracy in predicting the occurrence of *C. imicola* in Iberia. However, although the analysis may have identified the major environmental constraints, the small percentage of sites for which the presence or absence of *C. imicola* was incorrectly predicted suggests that there may be additional factors involved (e.g. presence of vertebrate hosts, soil type or other climatic variables). Nevertheless the sites in south-eastern Spain (e.g. Granada, Almeria and Alicante) where *C. imicola* was incorrectly predicted to occur, represent areas where *C. imicola* could expand into. Rawlings *et al.* (1998) and Baylis and Rawlings (1998) also identified this region as suitable for the invasion of *C. imicola* and it is possible that further, more intensive, sampling in this region may reveal its presence.

The impact of temperature on the distribution was not unexpected, as *C. imicola* reaches the northern limits of its global distribution in Iberia. The analysis indicates that the presence of *C. imicola* is favoured by high summer temperatures combined with mild winter temperatures, while the number of months with mean temperatures ≥12.5°C provides a measure of the likelihood of population persistence and growth. Thus it appears that the distribution of *C. imicola* is limited by two factors. First, the

severity of the winter, which will prevent adult and immature survival in some areas. Second, summer temperatures, which will influence the development and population growth rates and hence also the likelihood of the midges surviving the subsequent winter.

The application of the model to other European countries has provided useful insight into identifying where *C. imicola* could occur. In Greece, not only does the model predict that *C. imicola* could occur in some of the areas where it has already been found (e.g. Chaldithiki, Larisa, Chios, Lesbos and Samos), but also that it could occur in areas further south in mainland Greece and the islands of Andros, Corfu, Crete, Limnos, Naxos and Zante. However, the model does not account for topography and hence *C. imicola* may not necessarily occur at all these sites. For example, for *C. imicola* to reach Corfu and Zante, it would have to initially extend its range into western Greece but the Pindos Mountains in central Greece are likely to hinder this process. However, the spread of *C. imicola* south of Larisa is feasible and further sampling in this area may reveal its presence.

The occurrence of *C. imicola* in southern Italy is again largely dependent on its initial spread into western Greece, from where the adult midges could be carried on the wind into Italy. For example, the distance between Corfu and the heel of Italy is <200 km, which is well within the range of distances (i.e. up to 700 km) over which *Culicoides* can be carried on the wind (Sellers, 1992). The chances of *C. imicola* occurring in Albania are also largely dependent on its spread into western Greece. However, if *C. imicola* did reach Albania, it would be unlikely to spread further north into the coastal regions of Yugoslavia, Bosnia and Croatia for two reasons. First, the rainfall in these areas is likely to be too high (i.e. >1000 mm/year). Second, the mountains in Montenegro would present a physical barrier to the spread of the midges.

If the global mean temperature does increase by 2°C by 2100 (as predicted by climate change scenarios; Intergovernmental Panel on Climate Change, 1996), *C. imicola* could potentially extend its range even further in Europe. For example, in Spain the model indicates that *C. imicola* could occur further north and east. It is even possible that *C. imicola* could reach the south of France. In Greece, *C. imicola* could

potentially extend its range into the north-east of the country (e.g. Kavala and Alexandroupolis) and continue its southwards migration. In addition, if *C. imicola* ever reaches Italy or Albania, greater areas of these countries would have suitable climates for its occurrence.

However, while the model provides insight into the potential influence of global warming on the range of *C. imicola* in Europe, it must be borne in mind that the distribution of a species is affected by additional factors which were not incorporated into the model. For example, interactions with other species (e.g. competition and enemy-victim interactions) will influence distribution and Davis *et al.* (1998a,b) found that these interactions are also likely to vary with temperature. Consequently, the actual range of a species under warmer conditions may differ from that predicted from climate data alone. In addition, the model does not account for adaptation of *C. imicola* to the cooler temperature regimes, which could result in *C. imicola* exploiting even greater areas of Europe.

In this chapter, I have described a simple model for predicting the potential range of *C. imicola* in Europe, both currently and if conditions should warm with climate change. *Culicoides imicola* is the principal vector species of BTV and AHSV in Europe and hence it is essential to establish where it could occur. The model displayed a high degree of accuracy in predicting the occurrence of *C. imicola* in Iberia when compared with the published data (Rawlings *et al.*, 1997) and also indicated that *C. imicola* could potentially expand its distribution in Spain and Greece. In addition, if *C. imicola* can extend its range into western Greece, it may then be able to invade both Italy and Albania. Furthermore, while it must be borne in mind that there is a degree of uncertainty associated with predictions of distribution based on climate data alone, it is likely that with global warming the spread of *C. imicola* in Europe will be even more extensive than that predicted under current conditions.

CHAPTER 7: CONCLUSIONS, CONSEQUENCES AND PROSPECTS

In this final chapter I first summarise my results. I then discuss the current climate change scenarios and consider how these changes could alter the risk of bluetongue virus (BTV) and African horse sickness virus (AHSV) occurring in Europe. Finally, I consider methods for controlling the viral diseases and suggest areas for future research.

7.1 CONCLUSION

To assess the potential impact of climate change on *Culicoides*-transmitted viruses, it is essential first to understand how climatic factors affect the vector and the viruses. In this thesis I have attempted to quantify the impact of temperature on some aspects of virus transmission. For example, I have shown that temperature affects both the development rate and survivorship of immature *Culicoides*, which in turn influence the adult recruitment rate. The optimum temperatures for recruitment of adult midges were estimated to be 25-30°C for *C. nubeculosus* and 25-35°C for *C. variipennis sonorensis*, while the minimum temperatures for development were calculated as 8.1°C and 10.7°C respectively (Chapter 2). I have also shown that temperature can affect the proportion of adult *Culicoides* that could potentially transmit the viruses, through its impact on the duration of the viral extrinsic incubation period and adult survival. High temperatures (27-30°C) favour transmission of the viruses because although adult longevity is reduced (Chapter 3), the duration of the extrinsic incubation period is sufficiently shortened (Chapters 3 and 4) that a greater proportion of midges could survive to transmit virus. In contrast, at temperatures below the theoretical minimum for virus development (e.g. 7.6-15.1°C depending on the virus species and serotype; Chapters 3 and 4) virus transmission does not occur. However, if temperatures subsequently increase, viral development can recommence, increasing the potential for transmission (Chapter 3). I have also shown that the impact of temperature on the vector competence of *Culicoides* vector populations is variable (Chapters 3 and 4). For example, in some cases vector competence increased with temperature (e.g. *C. variipennis sonorensis* infected with AHSV4 or EHDV1), while in others, temperature had no effect on vector competence (e.g *C. variipennis sonorensis* infected with BTV10 or BTV16 and *C. imicola* infected with AHSV8). In

addition, I found that exposure of immature *C. nubeculosus* (a non-vector species) to temperatures close to their upper lethal limit (33°C), for the whole life cycle or just the pupal stage, could induce vector competence for BTV and AHSV (Chapter 5). Finally, I have shown that temperature is a significant factor limiting the distribution of *C. imicola* in Europe. With global warming, the potential spread of *C. imicola* in Europe is likely to be even more extensive than that predicted under current conditions (Chapter 6).

7.2 Consequences of Climate Change on BTV and AHSV

Climate change scenario

Climate models predict that the annual global mean surface air temperature will increase by 2°C by 2100, with an uncertainty range from 1.5 to 3.5°C (Intergovernmental Panel on Climate Change, 1996; Houghton, 1997). Relatively greater increases are expected in winter than in summer, and in night time versus day time temperatures. In addition, there is expected to be an increase in the frequency of very warm days coupled with a decrease in the number of very cold days. Spring is also expected to arrive earlier and autumn later, and across Europe, Menzel and Fabian (1999) have calculated that the average annual growing season has already lengthened by 10.8 days since the 1960s, due to increasing temperatures. The annual global mean precipitation is also predicted to increase, although some areas may get drier (Intergovernmental Panel on Climate Change, 1996). Additionally, changes in wind patterns are likely.

Consequences of climate change

Changes in vector distribution: Warmer temperatures are likely to increase the geographic range of many insect species (unless the upper lethal limit is exceeded; Gates, 1993) and climate changes in the past have been associated with shifts in insect distributions (Elias, 1994, 1995). Indeed some insect species are already responding to the anomalous climate of the 20th century. For example, in a survey of 35 non-migratory European butterfly species, Parmesan *et al.* (1999) found that 14 species had extended their range northwards by 35-240 km during the last 100 years.

Climate change is therefore likely to influence the distribution of *Culicoides* vector species. Of greatest concern with regards to BTV and AHSV in Europe is the potential

expansion in the range of *C. imicola*. The distribution of this species in Europe appears to be limited by low temperatures (Baylis and Rawlings, 1998; Chapter 6) and hence, as conditions warm, *C. imicola* will be able to exploit greater areas of this continent. *Culicoides imicola* is currently known to occur in south-western Iberia (Rawlings *et al.*, 1997), eastern Greece (Patakakkis, unpub. obs.) and several Greek islands (Boorman and Wilkinson, 1983; Boorman, 1986; Mellor pers. comm.; Patakakkis, unpub. obs). The areas at particular risk from the invasion of *C. imicola*, if conditions warm, include eastern Spain and north-eastern and southern Greece. Conditions in western Greece are also likely to be suitable for *C. imicola*, although its spread into this area may be hindered by the Pindos mountains in central Greece. However, if *C. imicola* can extend its range into western Greece, it may then be able to invade both Italy and Albania.

The expansion in range of *C. imicola* will increase the areas of Europe at risk of BTV and AHSV. In addition, the extended distribution of *C. imicola* could bring BTV and AHSV into the range of *C. obsoletus* and *C. pulicaris*. These species are potential vectors (Mellor and Pitzolis, 1979; Mellor *et al.*, 1990) and are much more widely distributed in Europe than *C. imicola*. Thus once infected it is possible that they could spread the viruses over even larger areas of Europe (Mellor and Boorman, 1995). Indeed this phenomenon may have been involved in the recent BTV outbreak in Bulgaria. For example, *C. imicola* is thought to be absent from Bulgaria, while *C. obsoletus* was the most numerous species trapped at infected sites (Mellor, pers. comm.). At the time of the outbreak, BTV was also reported from Greece and Turkey, where *C. imicola* and *C. obsoletus* coexist (Jennings *et al.*, 1983; Mellor *et al.*, 1984; Patakakkis, unpub. obs.). Hence it is possible that *C. obsoletus*, infected in these regions, were then able to spread the virus to more northerly populations of *C. obsoletus*, such as those in Bulgaria.

However, Europe is not the only continent likely to suffer from an increased risk of *Culicoides*-transmitted viruses due to climate induced changes in vector distribution. For example, in Australia BTV vector species *C. wadai* and *C. brevitarsis* are predicted to extend their distributions into some sheep and cattle rearing areas which are currently vector-free (Sutherst, 1990; Standfast and Maywald, 1992; Sutherst, 1993; Ward, 1994). In addition, a further 15 states in the USA are predicted to become

endemically infected with BTV (Gibbs *et al.*, 1989) due to the expansion in the range of the principal vector species *C. variipennis sonorensis*.

Changes in vectorial capacity: Climate change will also affect the vectorial capacity of *Culicoides* populations. For example, adult *Culicoides* are likely to become more abundant. Thus increasing temperatures coupled with an extension in the development season will result in a greater number of generations (and therefore adults) per year. In addition, the overwintering ability of adult *Culicoides* (as well as the immature stages of non-diapausing species) is likely to improve, as winters become both warmer and shorter. For example, Bishop *et al.* (1995) showed that a 2°C rise in winter temperatures could extend the last occurrence of adult *C. brevitarsis* by 0.7 months. Improved overwintering success is also likely to increase the spring population input, which in turn could result in larger populations during the summer.

Climate change will also affect the proportion of adult *Culicoides* capable of transmitting the viruses. For example, warmer temperatures will increase the likelihood of female *Culicoides* surviving long enough to blood-feed after completing the viral extrinsic incubation period. Vector competence will also be affected, as temperatures will be conducive for virus development for a greater proportion of the year. The impact of warmer temperatures on vector competence will be even greater for those virus-vector combinations where vector competence increases with temperature. In addition, the predicted increase in frequency and intensity of extremely warm days could result in the creation of new vector species (e.g. *C. nubeculosus* via the leaky midgut phenomenon).

The impact of climate change on vectorial capacity will have three main effects on BTV and AHSV transmission in Europe. First, the greater abundance of adult *Culicoides* combined with the increased proportion of females capable of transmitting virus will increase the risk of an epidemic occurring following the introduction of virus into an area. The greatest risk will be at times of the year when temperatures reach ≈25-30°C (i.e. when conditions are optimal for adult recruitment and virus transmission). Second, as temperatures will be conducive for both viral and *Culicoides* development for a greater proportion of the year, the length of the viral transmission season will increase. Third, the enhanced overwintering success of adult

Culicoides combined with the extension in the development season, will increase the seasonal occurrence of adult midges. In turn, this will improve the overwintering ability of the viruses.

In summary, it is likely that the influence of climate change on vector distribution and vectorial capacity will serve to extend the areas of Europe at risk of BTV and AHSV, as well as increasing the likelihood and severity of epidemics within these regions following the introduction of virus.

Other vector-borne diseases

The principles involved in the transmission of BTV and AHSV by *Culicoides* are similar to those of many other vector-borne diseases. Indeed, changes in the geographic distribution and seasonal occurrence of diseases such as malaria transmitted by anopheline mosquitoes (Martin and Lefebvre, 1995; Martens *et al.*, 1999), dengue and yellow fever transmitted by *Aedes aegypti* and *Ae. albopictus* (Shope, 1991), and sandfly fever and leishmaniasis transmitted by *Phlebotomus papatasi* (Cross and Hyams, 1996) are also predicted to occur with climate change.

7.3 PROSPECTS

BTV and AHSV pose an increasing threat to Europe during the 21st century and it is therefore vital that effective control methods are available. Control programmes generally include the vaccination of susceptible animals and at present live attentuated vaccines are available for use against both viruses. These are generated by adapting field isolates of the viruses to growth *in vitro* and serially passaging them in tissue culture cells. This process selects viruses that become increasingly adapted to growth *in vitro*, but which have a decreased pathogenicity for the vertebrate host.

However, while these vaccines are cheap to produce and provide a high level of protection in susceptible vertebrates, there are several undesirable side-effects. First, some attentuated BTV strains are teratogenic (Erasmus, 1990; Johnson *et al.*, 1992a,b) and pregnant ruminants treated with these vaccines may abort or produce young with congenital abnormalities. Second, there is concern that the vaccine viruses could revert to virulence. Indeed the death of cattle in the recent BTV outbreak in Rhodes (1999) has been attributed to this by some authorities (Anon, 2000a). Third, it is

possible that the attentuated viruses could reassort with field viruses, potentially resulting in the creation of new viral strains. Fourth, given that most animals develop a viraemia as a result of vaccination, there is concern that *Culicoides* vectors could transmit the vaccine viruses. Furthermore, following challenge with a virulent virus, some vaccinated animals may develop a viraemia, although clinical signs and death are prevented (House *et al.*, 1992). These animals would then be a covert source of virus for *Culicoides* vectors. Fifth, vaccinated animals cannot be distinguished serologically from those naturally infected with the viruses.

While these vaccines may be suitable for use in enzootic regions, where the principal aim is to reduce animal losses by protecting against disease, they are not necessarily appropriate for use in non-enzootic areas (such as Europe), where the goal is eradication. Ideally a vaccine which protects susceptible animals from disease, as well as preventing *Culicoides* vectors from becoming infected should be used. Consequently, given the increasing threat that BTV and AHSV pose to Europe, it would be advantageous to develop alternative vaccine strategies that satisfy these criteria.

Indeed, in the case of BTV (which has become the priority in view of the recent BTV outbreaks in Greece and Bulgaria), the European Union is considering two options. First, the development of inactivated whole virus vaccines. Although these vaccines are expensive to produce, they have a number of advantages. For example, they cannot revert to virulence or recombine with other viruses. They do not cause a viraemia in inoculated animals and therefore cannot infect *Culicoides* vectors. Further, House *et al.* (1994) found that horses treated with two doses of an inactivated AHS vaccine were prevented from developing a viraemia following exposure to a virulent virus strain. In addition, with further research it may be possible to differentiate between animals treated with inactivated vaccines and those naturally infected. For example, viral non-structural proteins (NS1, NS2 and NS3) can be removed during the viral purification procedure and assays that detect antibodies to these non-structural proteins can then be used to differentiate vaccinated from naturally infected animals. Second, there is the possibility of using subunit virus vaccines, which contain virus proteins but no viral genetic material. These vaccines may include those based on virus-like particles, which contain the core viral proteins VP3 and VP7 and the outer

capsid proteins VP2 and VP5, and core-like particles, which contain only the core proteins VP3 and VP7 (see Figure 1.2). These vaccines are also unable to revert to virulence, recombine with other viruses or be transmitted by insects. However, the development of commercial quantities of recombinant virus vaccines may prove to be difficult.

As well as developing alternative vaccines for the control of BTV and AHSV in Europe, future research should focus on the vectors. For example, in order to identify areas of Europe at risk from the viral diseases, it is essential to establish the full extent of the distribution of *C. imicola*. In particular, greater areas of Greece should be surveyed and it would also be advantageous to monitor the spread of *C. imicola* in Europe as conditions warm. In addition, the climatic requirements of this species should be defined more precisely. Identification of the parameters influencing the abundance and seasonality of *C. imicola* would also allow for the more accurate prediction of high or low risk years for the occurrence of the viruses. Finally, the role of the potential vector species *C. obsoletus* and *C. pulicaris* in the transmission of BTV and AHSV must be defined more clearly.

APPENDIX 1

Calculation of Viral Titres

Method for calculation of $TCID_{50}$ (i.e. dilution of virus required to infect 50% of cells) adapted from Spearman (1908) and Kärber (1931):

$$\text{Log}_{10}\,TCID_{50} = -\left(\begin{array}{c}\text{Highest dilution}\\ \text{giving 100\% CPE}\end{array}\right) + 0.5 + \left(\frac{\begin{array}{c}\text{No. of test units with CPE at dilutions}\\ \text{higher than that producing 100\% CPE}\end{array}}{\text{No. of test units per dilution}}\right)$$

Calculations

1.1 Pools of 5 *C. variipennis sonorensis* (Chapter 3)

- Pools of midges ground up in 0.5 ml of diluent
- 6 ten-fold dilutions prepared
- 0.1 ml of each dilution inoculated onto each of 6 microtitre plate wells (test units)

Example

Log_{10} virus dilution	Infected test units
-1	6/6
-2	6/6
-3	6/6
-4	4/6
-5	1/6
-6	0/6

$\text{Log}_{10}\,TCID_{50} = -(-3) + 0.5 + 5/6$

$= 4.333\ \log_{10} TCID_{50}/0.1$ ml (given that inoculation of virus dilution was 0.1 ml)

$= 1\ \log_{10} + 4.333\ \log_{10} TCID_{50}/\text{ml}$

$= 5.333\ \log_{10} TCID_{50}/\text{ml}$[a]

[a] To calculate the $\log_{10}$ $TCID_{50}$/5 midges, 0.3 $\log_{10}$ (= 2) was subtracted from this value, as the pools of midges were ground up in half the volume (i.e. 0.5 ml).

This assay could detect viral titres of 1.367 - 7.2 log_{10} $TCID_{50}$/5 midges. The first infected test unit (i.e. 1/6 infected test units, –1 log_{10} dilution) represented a titre of 1.367 log_{10} $TCID_{50}$/5 midges and each additional infected test unit represented a titre of 0.167 log_{10} $TCID_{50}$/5 midges.

1.2 Individual *C. variipennis sonorensis* (Chapter 3)

- Individual midges ground up in 0.5 ml of diluent
- 3 ten-fold dilutions prepared
- 0.1 ml of each dilution inoculated onto each of 5 microtitre plate wells (test units)

Example

Log_{10} virus dilution	Infected test units
-1	5/5
-2	2/5
-3	0/5

Log_{10} $TCID_{50}$ = -(-1) + 0.5 + 2/5

= 1.9 log_{10} $TCID_{50}$/0.1 ml (given that inoculation of virus dilution was 0.1 ml)

= 1 log_{10} + 1.9 log_{10} $TCID_{50}$/ml

= 2.9 log_{10} $TCID_{50}$/ml[b]

[b] To calculate the log_{10} $TCID_{50}$/midge, 0.3 log_{10} (= 2) was subtracted from this value, as individual midges were ground up in half the volume (i.e. 0.5 ml).

This assay could detect viral titres of 1.4 - 4.2 log_{10} $TCID_{50}$/midge. The first infected test unit (i.e. 1/5 infected test units, –1 log_{10} dilution) represented a titre of 1.4 log_{10} $TCID_{50}$/midge and each additional infected test unit represented a titre of 0.2 log_{10} $TCID_{50}$/midge.

1.3 Pools of 25 *C. imicola* (Chapter 4)

- Pools of midges ground up in 1 ml of diluent (midge suspension)
- 4 ten-fold dilutions prepared
- 0.1 ml of midge suspension and each dilution inoculated onto each of 4 microtitre plate wells (test units)

Example

Log_{10} virus dilution	Infected test units
0	4/4
-1	4/4
-2	4/4
-3	1/4
-4	0/4

$Log_{10}\ TCID_{50} = -(-2) + 0.5 + 1/4$

$= 2.75\ log_{10}\ TCID_{50}/0.1$ ml (given that inoculation of virus dilution was 0.1 ml)

$= 1\ log_{10} + 2.75\ log_{10}\ TCID_{50}/ml$

$= 3.75\ log_{10}\ TCID_{50}/ml^{c}$

[c] Since the pools of midges were ground up in 1 ml of diluent this value is equivalent to the $TCID_{50}$/25 midges.

This assay could detect viral titres of 0.75 - 5.5 log_{10} $TCID_{50}$/25 midges. The first infected test unit (i.e. 1/4 infected test units, undiluted midge suspension) represented a titre of 0.75 log_{10} $TCID_{50}$/25 midges and each additional infected test unit represented a titre of 0.25 log_{10} $TCID_{50}$/25 midges.

To calculate the lowest viral titres (i.e. 0.75-1.5 log_{10} $TCID_{50}$/25 midges; CPE only in test units containing undiluted midge suspension), for which there is no value for the highest dilution producing 100% CPE, 1 log_{10} was subtracted from the equivalent titres calculated for –1 log_{10} dilution (Table A.1).

Table A.1 Calculation of lowest viral titres i.e. 0.75-1.5 log_{10} $TCID_{50}$/25 midges.

Log_{10} virus dilution	Infected test units	Viral titre log_{10} $TCID_{50}$/25 midges
0	1/4	1.75 – 1 = 0.75
0	2/4	2 – 1 = 1
0	3/4	2.25 – 1 = 1.25
0	4/4	2.5 – 1 = 1.5
-1	1/4	1.75
-1	2/4	2
-1	3/4	2.25
-1	4/4	2.5

1.4 Individual *C. imicola* (Chapter 4)

- Individual midges ground up in 1 ml of diluent (midge suspension)
- 2 ten-fold dilutions prepared (if virus was initially detected in the undiluted midge suspension)
- 0.1 ml of midge suspension and each dilution inoculated onto each of 4 microtitre plate wells

Calculation of viral titres in individual *C. imicola* was the same as for the pools of 25 *C. imicola* (A3), except the assay could only detect viral titres of 0.75 - 3.5 log_{10} $TCID_{50}$/midge as only 2 ten-fold dilutions were prepared.

1.5 Individual *C. nubeculosus* (Chapter 5)

- Individual midges ground up in 0.5 ml of diluent
- 3 ten-fold dilutions prepared
- 0.1 ml of each dilution inoculated onto each of 6 microtitre plate wells (test units)

Example

Log_{10} virus dilution	Infected test units
-1	5/6
-2	2/6
-3	0/6

Log_{10} $TCID_{50}$ = -(-1) + 0.5 + 2/6

= 1.833 log_{10} $TCID_{50}$/0.1 ml (given that inoculation of virus dilution was 0.1 ml)

= 1 log_{10} + 1.833 log_{10} $TCID_{50}$/ml

= 2.833 log_{10} $TCID_{50}$/ml[d]

[d] To calculate the log_{10} $TCID_{50}$/midge, 0.3 log_{10} (= 2) was subtracted from this value, as individual midges were ground up in half the volume (i.e. 0.5 ml).

This assay could detect viral titres of 1.367 - 4.2 log_{10} $TCID_{50}$/midge. The first infected test unit (i.e. 1/6 infected test units, –1 log_{10} dilution) represented a titre of 1.367 log_{10} $TCID_{50}$/midge and each additional infected test unit represented a titre of 0.167 log_{10} $TCID_{50}$/midge.

APPENDIX 2

Calculation of Saturation Deficit (Chapters 3 and 6)

Saturation deficit mbar = SVP – AVP

SVP = saturation vapour pressure mbar, AVP = actual vapour pressure mbar

Calculation of SVP

$$SVP = 9.24349 - \frac{2305}{T} - \frac{500}{T^2} - \frac{100000}{T^3}$$

where T = temperature °K

Calculation of AVP

$$\text{Relative humidity (RH)} = \left(\frac{AVP}{SVP}\right) \times 100$$

$$\therefore AVP = \frac{RH}{100} \times SVP$$

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