STUDIES ON DROSOPHILA C AND A VIRUSES IN AUSTRALIAN POPULATIONS OF DROSOPHILA MELANOGASTER

A thesis submitted for the degree of Doctor of Philosophy of the Australian National University

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DECLARATION

The research carried out in the course of this investigation and the results presented in this thesis are, except where acknowledged, the original work of the author.

[Signature]

Peter Christian
November 1987
ABSTRACT

Two small RNA viruses, *Drosophila* A and C viruses (DAV and DCV) were isolated from laboratory populations of *D. melanogaster* established from wild-caught Australian flies. Initial evidence suggested that these viruses were likely to be encountered in natural populations of *D. melanogaster* in Australia. The isolated viruses proved to be serologically related to DAV and DCV previously isolated from *D. melanogaster* in Europe.

To facilitate further studies on the biology of DAV and DCV, and to ascertain their distribution in natural *Drosophila* communities in Australia, cDNA fragments of the RNA genomes of both viruses were cloned. Cloned cDNAs were then used to develop specific nucleic acid hybridization assays for both viruses. These assays proved capable of detecting as little as 1-10 ng of virus in isolates from single flies.

Laboratory studies revealed that DAV and DCV were tolerated in laboratory stocks of *D. melanogaster* as persistent, chronic infections. The presence of the viruses decreased the life-expectancy of adult flies derived from these persistently infected stocks, but the pathogenic effect was restricted to times more than 18 days post-eclosion. In addition, it was demonstrated that DAV was associated with preferential selection against pre-imago males and, indirectly via its pathogenic effect, with total egg-production between 4-20 days.

Individual *Drosophila* from 34 Australian localities spanning 26.5° of latitude were surveyed for the presence of DAV and DCV. Individuals from 8 localities were found to be infected with DAV and from 6 localities with DCV. Overall, 12 (35%) of the 34 communities screened contained at least one virus. DCV was detected in only *D. melanogaster* and *D. simulans*; DAV was also detected in these two species and in
D. immigrans and D. sulfurigaster. These data are discussed in relation to the major hosts for DAV and DCV in natural Drosophila communities, and the possible origins of the viruses in Australian Drosophila.

Within infected communities no difference was found in the frequency of DAV and DCV infection between D. melanogaster and D. simulans. Furthermore, there was no heterogeneity in the frequency of DCV infection between infected populations of D. melanogaster/simulans, and no geographical pattern could be identified in the distribution of populations infected with DCV.

Considerable heterogeneity was found in the infection frequencies within DAV-infected populations of D. melanogaster/simulans. This heterogeneity was due to the high frequency of infection found in two populations collected in 1984 and 1985 at Coffs Harbour. Reasons for the abnormally high frequencies at this site are discussed in the light of the available evidence on the biology and ecology of DAV and of Drosophila.

The modes of transmission found for DAV and DCV in laboratory populations of D. melanogaster are discussed in terms of their relative importance in natural populations. The effects of DAV and DCV infections in D. melanogaster are compared with those of other non-occluded viruses in insects, and the interaction between the biology and ecology of the viruses and their hosts is discussed.
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Finally, I can never hope to express my thanks deeply enough to my Mum and Dad for their continual support, encouragement and love through the years.
1.1 INTRODUCTION

The insects comprise about three-quarters of the world’s species of animals. During the course of their evolution they have become adapted to a wide range of habitats. These range from high latitudes to the equator, rainforests to deserts and from high altitudes to sea-level. At the same time they have acquired varied life-habits - including, phytophagous, saprophagous, parasitic and carnivorous types. Among the insect orders (Figure 1.1) the Diptera are one of the largest, and with an estimated 100,000 known species (Wheeler, 1981) they comprise about 10% of the world’s total insect fauna while also reflecting much of their ecological diversity.

Within the Diptera is a family of small to medium sized flies, usually 2.5 - 3.0 mm in length, with a body colour of various shades of yellow, tan, light to dark brown and black. These are the Drosophilidae, of which Drosophila melanogaster is perhaps the best known species.

Since the turn of the century and its first uses as a laboratory animal, research with D. melanogaster, and later on other members of the genus Drosophila, has made major contributions to our knowledge of many areas of biology. Despite this immense research effort, relatively little is known of the viruses of Drosophila and of the interactions between these viruses and their hosts. In particular, compared with other viruses of insects, most of the viruses of Drosophila have been little studied in terms of their distribution within and between natural populations and the possible mechanisms by which they are maintained within populations. This lack of information is principally due to the technical difficulty in the detection of these viruses. However, developments in the technology of virus
Figure 1.1 The orders comprising the Insecta, and a suggested phylogeny, after Mackerass (1970).

<table>
<thead>
<tr>
<th>SUBCLASS</th>
<th>INFRACLASS</th>
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detection over the past ten years have to a great extent circumvented these technical problems.

There are four features of the genus Drosophila, especially in Australia, that makes it an interesting group in which to study its viruses and in particular their biology and ecology:

(i) a number of species of Drosophila may be found together at any one collecting site where they form a community of closely related species. This provides an opportunity to study the virus-host specificity of a virus under natural conditions.

(ii) within the genus there are a number of species that are considered to be cosmopolitan in their distribution (Patterson and Stone, 1952). Most of these species are to be found in Australia where they have undoubtedly been introduced by man. Since their introduction these species have remained associated with man-made habitats, while some endemic species have subsequently adapted to these habitats e.g. urban rubbish dumps, banana plantations and vineyards. These communities therefore form points of contact between native and introduced species within the genus. Where this contact occurs it provides an opportunity to study whether the viruses of the introduced species have become adapted to the endemic species and vice versa.

(iii) the eastern seaboard of Australia ranges in latitude from 10.5°S. to 38°S. (44°S. including Tasmania) and therefore provides a potentially continuous distribution for Drosophila communities. However, the ecological diversity inherent within these latitudes means that for any one species to be continuously distributed over this range it would need to adopt a variety of life-history strategies dependant on the prevailing environment. This affords a unique opportunity to
examine the capacity of viruses to co-adapt to the life-
history strategy of their host.

(iv) the distribution of Drosophila species does vary with latitude
within Australia and for two of the cosmopolitan species there
is evidence that populations have become genetically
differentiated latitudinally. This offers an opportunity to
study the distribution of viruses in natural populations of a
species and to determine whether it reflects any of the
latitudinal clines previously described.

D. melanogaster was chosen as a working species for this project
for several reasons. Firstly, most of the viruses isolated previously
from members of the Drosophila have been isolated from this species.
Secondly, it is widely distributed down the eastern seaboard of
Australia and occurs in association with different groups of species
in different geographic localities. Finally, it is one of the
cosmopolitan species mentioned above that shows latitudinal genetic
differentiation. For these reasons it was decided that the main aims
of this thesis were to be:

(i) to isolate and characterise the viruses present in natural
populations of D. melanogaster,

(ii) to develop a sensitive and specific assay system for detection
of the common virus(es) found in these populations,

(iii) to ascertain the distribution of the virus(es) both within and
between natural Drosophila communities, and

(iv) to investigate the biology of the virus(es) and the possible
effects that they might have on natural populations.

The following literature review gives a brief survey of the range
of viruses to be found in insects, followed by a summary of the
information available on the biophysical/biochemical properties and
the biology and ecology of the viruses of Drosophila. The final
section of this chapter will deal with the Drosophila fauna of Australia concentrating on features pertinent to this study.

1.2 THE VIRUSES OF INSECTS

1.2.1. Introduction

A brief survey of the viruses of insects is given here which will include over-views of their structure and their biophysical and biochemical properties. The aim of this section is however, to provide a picture of the type of properties that are used to differentiate between viruses in general, and the insect viruses in particular. This review therefore, does not attempt a complete description and references are cited which provide more detail on certain groups of viruses.

Since the first description of the causative agent of an insect viral disease in 1856 by Maestri and Cornalia there have been many hundreds of viruses isolated from insects. In the last report of the International Committee on Taxonomy of Viruses (ICTV) 11 of the 20 families of viruses of animals were recognised as infecting insects (Matthews, 1982).

The viruses within these 11 families show a great diversity in the nature of their relationship with the insect. The first type of relationship is one where the insect acts merely as a passive vector of a virus of plants (e.g. the potyviruses) or animals (e.g. myxoma poxvirus). Secondly, there are viruses for which the insect is one of two alternating hosts; unlike the first case, the virus is able to multiply in the insect host in these instances. Examples of viruses such as these are the togaviruses and bunyaviruses of mosquitoes and vertebrates and the phytoreoviruses of leafhoppers and plants. The third type of relationship is where the virus infects only particular
insects, and often causes disease in those insects. It is this type of
virus that I will refer to as an insect virus.

The basic properties of the insect viruses and their distribution
within the *Insecta* and across the animal and plant kingdoms is
summarized in Table 1.1.

1.2.2 The Occluded Insect Viruses

Within the insect viruses the majority of isolates described are
of types that have their virions encapsulated during replication into
a virally-encoded pseudo-crystalline protein matrix known as the
polyhedra or inclusion/occlusion body. Viruses of this sort are
restricted to invertebrates, primarily insects, and include the
baculoviruses, the cytoplasmic polyhedrosis viruses (CPVs) and the
entomopoxviruses (EPVs).

The Baculoviridae are a family of viruses that include the nuclear
polyhedrosis viruses (NPVs), the granulosis viruses (GVs) and an
assortment of non-occluded nuclear viruses. The family is
classified by having virions that contain one or more nucleocapsids
enclosed within a single envelope, are bacilliform in shape and
measure about 40-60nm x 200-400nm. The genome consists of a single
molecule of circular double-stranded (ds) DNA of 58-110 x 10^6 daltons.
Replication is almost exclusively in the nucleus, the only exceptions
being some of the GVs that appear to replicate in the cytoplasm. The
final stages of replication involve the inclusion of the virions into
the polyhedral body. Unlike other occluded viruses, the polyhedra are
further enveloped in a host encoded envelope composed primarily of
mucopolysaccharides.

The CPVs are a genus within the family Reoviridae, a family which
includes viruses of vertebrates, invertebrates and plants. The CPVs
have icosahedral virions that are approximately 60nm in diameter and
Table 1.1 Some properties of the major groups of Insect viruses.

(a) The main sources of information were Harrap and Payne (1979), Moore and Tinsley (1982) and Longworth (1978)

(b) Family names/groups and generic divisions are as designated by Matthews (1982)

(c) The nature of the nucleic acid comprising the genome of the virus and the number of segments in each genome are given.

(d) Host orders were obtained from the references given in (a) and (b) except for the additional orders:

(1) Larsson, 1984
(2) Clark, 1982
(3) Eley et al., 1987
(4) Kim and Scott, 1978
(5) Paliwal, 1979
(6) D’Arcy et al., 1981a

(e) N = no hosts known except for invertebrates
V = vertebrate hosts also known
P = plant hosts also known

(f) As these viruses have not been characterized in sufficient detail to allow them to be placed into any of the existing groups of viruses it is unknown whether they have any counterparts among the vertebrate and plant viruses.
Table 1.1. Some properties of the major groups of Insect viruses.

<table>
<thead>
<tr>
<th>Family/Group (b)</th>
<th>Genera (b)</th>
<th>Abbreviation</th>
<th>Inclusion Body</th>
<th>Nucleic Acid/Segments (c)</th>
<th>Particle Symmetry</th>
<th>Envelope</th>
<th>Insect Host Orders (d)</th>
<th>Other Hosts (e)</th>
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<td>Helical</td>
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<td></td>
<td>B. Granulosis Virus</td>
<td>GV</td>
<td>+</td>
<td>ds DNA one</td>
<td>Helical</td>
<td>+</td>
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<tr>
<td></td>
<td>C.</td>
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<td></td>
<td>D.</td>
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* These four genera comprise the subfamily Entomopoxvirinae (Matthews, 1982)
<table>
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<tr>
<th>Family/Group&lt;sup&gt;(b)&lt;/sup&gt;</th>
<th>Genera&lt;sup&gt;(b)&lt;/sup&gt;</th>
<th>Abbreviation</th>
<th>Inclusion Body</th>
<th>Nucleic Acid/Segments&lt;sup&gt;(c)&lt;/sup&gt;</th>
<th>Particle Symmetry</th>
<th>Envelope</th>
<th>Insect Host Orders&lt;sup&gt;(d)&lt;/sup&gt;</th>
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<td></td>
<td>Undesignated</td>
<td></td>
<td>-</td>
<td>ss RNA one</td>
<td>Helical</td>
<td>+</td>
<td>Diptera</td>
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<tr>
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<td>Birnavirus</td>
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<tr>
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(continued.....)
Table 1.1...continued.

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<th>Family/Group (b)</th>
<th>Genera (b)</th>
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<tr>
<td>Nudaurelia-β Virus-Group</td>
<td>-</td>
<td>-</td>
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<td>ss RNA one</td>
<td>Isometric</td>
<td>-</td>
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<tr>
<td>Unclassified Small RNA Containing Viruses</td>
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<td>SRV</td>
<td>-</td>
<td>ss RNA one/unknown</td>
<td>Isometric</td>
<td>-</td>
<td>Diptera Hymenoptera Orthoptera (c) Hemiptera Coleoptera</td>
<td>N (f)</td>
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</tbody>
</table>
have characteristic "spikes" or projections at the vertices of the particle. Virions contain 10 segments of dsRNA with a total molecular weight in the region of 10-20 \times 10^6 daltons. Viral replication and the assembly of the polyhedra is, with rare exceptions, confined to the cytoplasm. Most CPV infections are restricted to the midgut epithelium of susceptible hosts. One feature of CPV replication, and also of the ungrouped reovirus-like particles, is the production of a large "virogenic-stroma" in the cytoplasm of infected cells which seems to be the site of virus/polyhedra assembly.

The EPVs are a sister group to the Chordopoxvirinae within the family Poxviridae. The subfamily (Entomopoxvirinae) contains 3 major genera whose division is based on morphological and host-range criteria. The virions are enveloped and are either "brick-shaped" or ovoid and measure in the range of 170-250nm x 300-400nm. They contain a single linear molecule of dsDNA of molecular weight 140-240 \times 10^6 daltons. Viral replication is in the cytoplasm of infected cells (mainly leucocyte and adipose cells), and as with the previous groups described, the final stages of replication involve the occlusion of the virions into the polyhedral body - usually termed the spherule in the case of EPVs.

1.2.3 The Non-Occluded Insect Viruses
1.2.3.1 The Viruses with DNA Genomes

The non-occluded insect viruses contain a wide variety of virus-types and really do not form a natural grouping on the basis of morphology, replicative strategy or any other criteria. As such, they have been treated in many different ways by reviewers (see for example, Longworth, 1978 and Moore and Tinsley, 1982) who invariably fail to encompass all of the groups. For the purpose of this review I have chosen to separate the non-occluded viruses into those having DNA and those having RNA genomes.
The DNA-containing viruses therefore include the Iridoviridae and the Parvoviridae. Matthews (1982) recognized two genera of iridoviruses, Iridovirus and Chlor iridovirus, which infect insects. Iridovirus infections in insects invariably produce vast numbers of virus particles which spontaneously crystallise in infected tissues to give a characteristic iridescent appearance. It is from this that the name iridovirus derives. Although the two genera are separated primarily on the size of the virions (Iridovirus virions measure 120nm in diameter compared to Chloriridovirus which measure 180nm in diameter) each genus is also characterised by producing a different coloured iridescence, blue in the former and green in the latter. The virions are icosahedral in shape and non-enveloped although, in some cases the virus acquires an envelope during its release, by budding, from the host cell membranes. However, most virus remains cell-associated and the unenveloped virions are infectious. Virions contain a single molecule of dsDNA which is 100-250 x 10^6 dalton in weight.

The Paroviridae are unique amongst the insect viruses as they are the only group that have a single-stranded (ss) DNA genome - a feature shared only with two groups of plant viruses and two groups of bacterial viruses. Paroviruses of insects have been placed in a separate genus from the vertebrate Paroviruses and are termed Densovirus. The virions are, as the name suggests, small - in the range of 20-25nm in diameter - and are nonenveloped and isometric. The ssDNA genome has a molecular weight of between 3.5 and 5.7 x 10^6 daltons. Mature virus particles contain either positive or negative strand DNA which are complementary and will form duplexes in vitro.

1.2.3.2 The Viruses with RNA Genomes.

The non-occluded RNA-containing viruses of insects represent a wide range of apparently unrelated viruses and as such have been grouped together here for convenience.
There are many reports in the literature of reovirus-like particles in the tissues of insects e.g. Plus et al., (1981), Kitajima et al., (1985), Eley et al., (1987). They are usually described as reovirus-like because they have the characteristic double-shelled structure of typical reovirus particles and a diameter of between 55nm and 80nm and lack a lipid envelope. In some cases it has been demonstrated that the particles contain ten (Boccardo et al., 1980) or eleven (Eley et al., 1987) segments of dsRNA - another characteristic of the typical reoviruses. All of these reovirus-like particles remain relatively uncharacterised and are as yet unassigned to genera. The taxonomy of these viruses is uncertain as there are groups of reoviruses that infect insects and vertebrates or plants alternately, and are able to multiply in both hosts.

The rhabdoviruses present a similar problem to that of the reoviruses in that there are groups within the family that are known to infect insects and either vertebrates or plants. Consequently, although there are descriptions of rhabdovirus-like particles that replicate in invertebrates and have no other known host e.g. Boccardo et al., (1980), the only good example of a rhabdovirus that is confined to insects is sigma virus of D. melanogaster. This virus will be discussed in detail in later sections.

Moore and Tinsley (1982) recognised six groups of small non-enveloped RNA viruses of insects:-

(i) picornaviruses

(ii) Nudaurelia β-like viruses

(iii) nodaviruses

(iv) birnaviruses

(v) bee chronic paralysis virus group

(iv) undefined viruses that have unusual properties or have been insufficiently characterised.
As all of the viruses isolated from Drosophila, with the exception of sigma virus, fall into one or other of these groupings, these will be discussed in more detail than the other groups of insect viruses.

Picornaviruses are a family of viruses with small particles, 22-30nm in diameter. The particles are icosahedral and have no visible core or surface projections. The genome consists of a single molecule of positive sense RNA that is polyadenylated at the 3' end and has a molecular weight of approximately $2.5 \times 10^6$ daltons. Furthermore, there is a small protein covalently attached to the 5' end of the RNA, referred to as the VPg. The virions contain four major polypeptides, three of which range in size between $24-41 \times 10^6$ daltons and one of $5.5-13.5 \times 10^6$ daltons. All of the proteins are produced by cleavage of a single polyprotein translated from the full length positive sense RNA.

Four genera have been recognised within the Picornaviridae based on differences in their pH-dependent stability and buoyant density: Enterovirus, Cardiovirus, Rhinovirus and Aphthovirus. Three insect viruses have been included in the family although they have not been assigned to genera (Matthews, 1982). These are Gonometa virus of G. podocarpi (Harrap et al, 1966), cricket paralysis virus (CrPV) of the field crickets Teleogryllus oceanicus and T. commudus (Reinganum et al, 1970) and Drosophila C virus (DCV) of D. melanogaster (Jousset et al, 1972). Each of these viruses possesses the biophysical properties that should place them in the genus Enterovirus (Longworth et al, 1973; Jousset et al, 1977; Longworth, 1978) and many workers have in fact treated them as such e.g. Moore and Tinsley (1982). More importantly, despite the disparate geographical origins of CrPV and DCV, they are serologically related (Reinganum and Scotti, 1976). The relationship between CrPV and DCV will be discussed further in section 1.3.2.
The Nudaurelia-β group of viruses has as its type member a virus isolated from the pine emperor moth Nudaurelia cytherea capensis from South Africa (Juckes, 1970). The virus particles of this group are icosahedral and have a diameter of approximately 35nm. The features that distinguish this group of viruses from other small icosahedral RNA viruses are:

(i) a single molecule of ssRNA of molecular weight $1.8 \times 10^6$ daltons which is not polyadenylated,

(ii) a single virion polypeptide with molecular weight of 60,000 daltons,

(iii) buoyant densities of between 1.28 and 1.30g/ml and sedimentation coefficients of 190-210 S.,

All members of the group which have been isolated to date have come from lepidopteran hosts and have been identified by their reaction with anti-sera against Nudaurelia-β virus. It is possible however, that a subgroup/genus within the family exists. A virus isolated from the soybean looper Pseudoplusia includens, although having only a single virion protein and a single RNA species is not serologically related to members of the Nudaurelia-β serogroup (Chao et al., 1983). It has a significantly different buoyant density and a structural protein of different molecular weight.

The first nodavirus was isolated from mosquitoes (Culex tritaeniorhynchus) from Japan (Scherer and Hurlbut, 1967) and was described as an arthropod borne picornavirus (Murphy et al., 1970). Later studies showed that the virus replicated in a variety of insects as well as in mosquito and baby hamster kidney (BHK) cell culture and it was also able to replicate and cause mortality in suckling mice (Bailey and Scott, 1973; Bailey et al., 1975; Tesh, 1980). The size of the virions (29-30nm), the buoyant density (1.34 g/ml) and the sedimentation coefficient (135S) suggested that it might be a
picornavirus until Newman and Brown (1975) demonstrated that the virions contained two RNA species of molecular weights $1 \times 10^6$ and $0.5 \times 10^6$ daltons. This showed it to be unrelated to the picornaviruses, and on the basis of the properties described, a new group of viruses was formed. The virions contain one major polypeptide of approximately 40,000 daltons and two minor proteins of molecular weights between 33,000 and 43,000 daltons.

Further members of the family have since been isolated from the New Zealand black beetle *Heteronychus arator* (Longworth and Archibald, 1975) and the grass grub *Costelytra zealandica* (Dearing et al., 1980). Other possible members also exist in this group, including Arkansas bee virus (Bailey and Woods, 1974) and an endogenous virus of *Drosophila* cells (Friesen et al., 1980).

The Birnaviruses or bi-segmented RNA animal virus group are a group of viruses that mainly infect fish, molluscs and birds. The type member of the group is infectious pancreatic necrosis virus of fish. The virus particles are icosahedral and measure approximately 60nm in diameter with no surface features and a core of about 45nm. The genome consists of two molecules of dsRNA of approximate molecular weights of $2.5 \times 10^6$ and $2.3 \times 10^6$ daltons. The only known isolates from insects are *Drosophila* X virus (Teninges et al., 1979) found as a contaminant of *D. melanogaster*, and a virus from a midge of the genus Culicoides (Miahle et al., 1983). Further properties of these viruses will be discussed in section 1.3.

The chronic bee paralysis virus (CBPV) group of viruses contains two members, CBPV of honeybees (Bailey et al., 1968) and RS virus of *Drosophila* of the *montium* and *ananassae* subgroups from Singapore (Plus et al., 1975a). Another possible member is a virus seen in tissues of the sheep blowfly *Lucilia cuprina* (Binnington et al., 1987). Viruses of this group have lemon-shaped particles and a size of $22-33 \text{nm} \times 30-$
65nm. CBPV virions contain one major polypeptide of molecular weight 23,500 daltons, whereas RS virus contains two proteins with molecular weights 41,000 and 19,500 daltons. Both viruses contain RNA but there is no information on the nature of these RNAs. RS virus will be discussed further in section 1.3.

The final "group" of viruses includes all of those small RNA viruses that have unusual properties that preclude their inclusion within any of the groups discussed above, or that have been insufficiently characterised to be placed into any existing groups. This "group" is very large and probably contains more viruses than all of the small RNA viruses of insects that have been placed in the groups outlined above.

1.3 THE VIRUSES OF DROSOPHILA

1.3.1 Introduction

In the late 1930s a CO₂ sensitive phenotype was described in laboratory stocks of D. melanogaster. The phenotype was later found to be cytoplasmically inherited and to have many properties of a virus (see L’Heritier, 1958). The agent responsible for the phenotype was tentatively termed "virus sigma". It was not until nearly thirty years after the discovery of the phenotype that the virus particles were observed for the first time in the electron microscope (Berkaloff et al., 1965).

Since the first observation of sigma virus particles, a further eight viruses have been isolated from wild and laboratory stocks of Drosophila. Unlike sigma virus, these viruses were not identified by any particular phenotype or pathological symptoms, but by the passaging of homogenates of apparently healthy flies in virus-free strains of D. melanogaster.
Of these viruses the most common types are picorna-like viruses (Drosophila P, C and A viruses and iota virus) (Plus and Duthoit, 1969; Jousset et al., 1972; Plus et al., 1975b; Jousset, 1970). These picorna-like viruses belong to three different serogroups (Plus et al., 1975b). Additionally, there is a reo-like virus, Drosophila F virus (Plus et al., 1975a), a virus of an unknown group called virus G (Plus et al., 1975a), a virus with unusual lemon-shaped virions called RS virus (Plus et al., 1975a) and the birnavirus, DXV (Teninges et al., 1979).

1.3.2 Biophysical and Biochemical Properties of Drosophila Viruses

Sigma virus is to date the only rhabdovirus of insects identified i.e. although there are many mammalian and plant rhabdoviruses that are transmitted by, and replicate in insects, sigma virus is the only virus that appears to have no host other than its insect host. It has been classified as a rhabdovirus primarily on the basis of its morphology and its similarities in this respect to vesicular stomatitis virus (VSV) (Berkaloff et al., 1965). Sigma virus has bullet shaped particles with a diameter of 70nm and a length of approximately 180nm. It has a spiculate coat and a spiral nucleocapsid of about 30 turns.

Sigma, because it is non-pathogenic and labile, has proved difficult to study at the biochemical and biophysical level. However, the virus does grow in Drosophila cell cultures (Ohanessian and Echalier, 1967). By utilising such cultures in combination with gentle purification procedures, Richard-Molner et al. (1984) showed that its virions contained five major polypeptides of molecular weights $210 \times 10^3$ (p210), $68 \times 10^3$ (p68), $57 \times 10^3$ (p57), $44 \times 10^3$ (p44) and $25 \times 10^3$ (p25) daltons.
The VSV group of vertebrate rhabdoviruses (Vesiculovirus) have virion proteins of molecular weights $150 \times 10^3$ (L), $70-80 \times 10^3$ (G), $50-62 \times 10^3$ (N), $40-50 \times 10^3$ (NS) and $20-30 \times 10^3$ (M) daltons; while viruses from the rabies virus group (Lyssavirus) contain 5 proteins of molecular weights $190 \times 10^3$ (L), $65-80 \times 10^3$ (G), $58-62 \times 10^3$ (N), $35-40 \times 10^3$ ($M_1$) and $22-25 \times 10^3$ ($M_2$) daltons. The p68 protein of sigma is, like the G protein of the vertebrate rhabdoviruses, glycosylated.

However, none of the sigma virus proteins were found to be phosphorylated whereas, the NS protein in vesiculoviruses and the N protein in lyssaviruses are heavily phosphorylated. Furthermore, the G protein of sigma virus particles can be removed by treatment with an anionic detergent which leaves them spikeless. This property is also to be found in the vertebrate rhabdoviruses.

Although many of the features of sigma virus differ slightly from those of the vertebrate rhabdoviruses, these features together with the morphology of the virions, demonstrate unambiguously that sigma is a rhabdovirus. Exactly how closely sigma virus is related to the vertebrate and plant rhabdoviruses is yet to be established.

The four picorna-like viruses of Drosophila can all be distinguished on the basis of their serogroup, buoyant density in neutral CsCl and by their major capsid proteins (see Table 1.2). Iota virus, originally isolated from D. immigrans, belongs to the same serogroup as DPV. However, the two viruses can be distinguished by the fact that iota virus, but not DPV, will induce $CO_2$ sensitivity when injected into D. melanogaster males (Jousset, 1972).

DCV is able to grow in Drosophila cell culture and so has consequently been the most studied of the picorna-like viruses of Drosophila. Along with CrPV and Gonometa virus, DCV has been placed into the Picornaviridae (see section 1.2.3.2.). The biophysical and biochemical properties of these three viruses would align them most
Table 1.2 Biochemical and biophysical properties of Drosophila picorna-like viruses compared with CrPV, Gonometa virus and poliovirus.

(b) Presence of a poly(A) tract at the 3' end of the genomic RNA.
(c) Presence of a small protein (VPg) covalently attached at the 5' end of the genomic RNA.
(d) Minor amount of protein present, assumed to correspond to VP₀ of mammalian picornaviruses.
(e) Molecular weights are from Jousset et al. (1977) but they vary with different isolates (see Plus et al., 1978; Scotti et al., 1981 and Moore et al., 1982)
(f) RNA shown to be present
(g) RNA found to be single-stranded but no molecular weight determined.

ND = Not determined.
Table 1.2 Biophysical and biochemical properties of Drosophila picorna-like viruses compared with CrPV, Gonometa virus and poliovirus.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Diameter (nm)</th>
<th>Mol. Wt. RNA (x10^-6)</th>
<th>Poly(A)(b)</th>
<th>VPG</th>
<th>Mol. Wt. Capsid Proteins x10^-3 daltons</th>
<th>Buoyant density in CsCl (g/ml) at pH 3</th>
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<tr>
<td>DCV</td>
<td>27</td>
<td>2.5-3</td>
<td>+</td>
<td>+</td>
<td>31,30,28,8.5(e),(37)(d)</td>
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<tr>
<td>DAV</td>
<td>25-30</td>
<td>2.5-3.0</td>
<td>ND</td>
<td>ND</td>
<td>72.9,41.2,31.6</td>
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<td>DPV</td>
<td>25-30</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>48,29.4,26</td>
<td>1.36</td>
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<tr>
<td>CrPV</td>
<td>27</td>
<td>2.5-2.8</td>
<td>+</td>
<td>ND</td>
<td>35,34,30,(43)(d)</td>
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<td>Gonometa Virus</td>
<td>32</td>
<td>ss(g)</td>
<td>ND</td>
<td>ND</td>
<td>36.5,32,29,12,(47.5)(d)</td>
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<td>Poliovirus</td>
<td>27-28</td>
<td>2.4</td>
<td>+</td>
<td>+</td>
<td>35,28,24,5.5,(41)(d)</td>
<td>1.34</td>
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</table>
closely with the picornavirus genus *Enterovirus* (Type Member, poliovirus) (see Table 1.2).

The replicative strategies of both DCV and CrPV are reminiscent of those of the mammalian picornaviruses (see Moore et al., 1985 for review). The RNA of both viruses is infectious and acts as a polycistronic mRNA. No subgenomic RNAs are produced and the only other detectable RNA is a high molecular weight ds RNA which is probably a replicative intermediate. The RNA is translated as a polyprotein which is then cleaved to form the capsid and non-structural polypeptides. CrPV has been demonstrated to encode an inhibitor-resistant protease, but processing of the the high molecular weight capsid protein precursors occurs more rapidly in the presence of *Drosophila* cellular proteases. One feature of CrPV and DCV not shared with the mammalian picornaviruses is that the capsid proteins are produced in supramolar excess. As no sub-genomic RNAs are detectable, regulation of the capsid protein precursor production is thought to occur at the translational level (see Moore et al., 1985).

CrPV has been shown to have a genome organisation similar to that of the mammalian picornaviruses. The capsid proteins are encoded at the 5' end of the genome and the non-structural proteins at the 3' end (Moore et al., 1985).

DCV and CrPV are obviously closely related at the biochemical and biophysical levels. *In vitro* they are even able to catalyse the processing of each others translational products (Reavy and Moore, 1983). Furthermore they are serologically related (Reinganum and Scotti et al., 1976; Plus et al., 1978). Even so, the serological relationship is not one of complete identity, and antisera against some strains of DCV will not react with CrPV unless the antigen concentration is raised 100 fold above that normally necessary to produce a reaction.
Using ribonuclease T₁ fingerprinting, Pullin et al. (1982) demonstrated that DCV and CrPV share no large oligonucleotides. Ribonuclease T₁ fingerprinting however, only detects approximately 15% of the sequence variation. Using cDNA hybridization analysis King et al. (1984) showed that there is no detectable sequence homology between DCV and CrPV. The seemingly close relationship of these two viruses obviously does not extend to the nucleotide level.

DCV has been isolated from a number of *D. melanogaster* populations from Morocco (DCV₀, DCVₜ and DCVₜ), France (DCV₉, DCV₇ and DCV₇) and the French Antilles (DCVₐ). All of these isolates are serologically indistinguishable but can, to a certain extent, be separated on the basis of their pathogenicity and host range (Plus et al., 1978). A comparison of seven isolates of DCV (Moore et al., 1982) showed that individual isolates could be separated by the proteins they induced in infected *Drosophila* cells. Ribonuclease T₁ fingerprinting of six different isolates indicated that while they all shared large oligonucleotides they could be distinguished by their smaller oligonucleotides (Clewley et al., 1983). cDNA hybridization analysis showed that DCVₜ, DCV₀, DCV₉ and DCV₇ have 60-80% homology between strains (King et al., 1984). All of these comparative studies of different geographical isolates of DCV have shown that the French isolates are more closely related to one another than they are to other isolates.

*Drosophila* P and A viruses are not pathogenic when injected into *D. melanogaster* and, although isolated from permanent cell lines have not been found to produce any noticeable cytopathic effect. Consequently they have not been studied at the biophysical and biochemical levels in as much detail as DCV. What is known of the biophysical and biochemical properties of these two viruses though shows them to be quite different from DCV and CrPV (Table 1.2). Their
high buoyant densities and the molecular weights of their capsid proteins has been most often cited as the reason for their exclusion from the Picornaviridae (see Moore et al., 1985). However, the properties of DPV are not that dissimilar from those of the Kashmir bee virus complex (Bailey et al., 1979) and bee slow paralysis virus (Bailey, 1976). Further characterisation at the biophysical and biochemical level should reveal whether these aberrant insect picornavirus-like viruses are indeed unusual picornaviruses or whether they belong to new groups of viruses.

Virus G was initially isolated from D. melanogaster from French Guyana and Drosophila of the montium and ananassae subgroups from Singapore (Plus et al., 1975a). The known biophysical and biochemical properties of this virus are summarized in Table 1.3. Its morphology and the size of its capsid proteins are unlike those of any other known insect virus.

RS virus was originally isolated from Drosophila from Singapore. Chronic bee paralysis virus (CBPV) and RS virus have been grouped together on the basis of their virion morphology (Moore and Tinsley, 1982). Nevertheless, RS virus and CBPV are distinct viruses. There is no serological relationship between the viruses and they have a different size range of capsid polypeptides and different buoyant densities. The known biophysical and biochemical properties of RS virus are given in Table 1.3.

Drosophila X virus (DXV) was initially isolated from D. melanogaster in a controlled passage in an experiment on sigma virus (Teninges et al., 1979). Plus (1978, 1980) found that the virus was a common contaminant of D. melanogaster cell lines and could also be isolated from passaging commercial foetal calf serum (FCS) in D. melanogaster flies. This evidence coupled with the fact that DXV has never been isolated from natural populations of flies seems to
Table 1.3 Biophysical and Biochemical Properties of Virus G, RS Virus, DXV and DFV (a)

<table>
<thead>
<tr>
<th></th>
<th>Size (nm)</th>
<th>Nucleic Acid</th>
<th>Mol. Wt. Virion Proteins $\times 10^{-3}$</th>
<th>Buoyant Density in CsCl (g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus G</td>
<td>25 (c)</td>
<td>RNA</td>
<td>58, 50, 43</td>
<td>1.34</td>
</tr>
<tr>
<td>RS Virus</td>
<td>32x50</td>
<td>RNA</td>
<td>45, 19.5</td>
<td>1.26</td>
</tr>
<tr>
<td>DXV</td>
<td>59</td>
<td>2 segments ds RNA 100, 45, 35, 25</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.6, 2.5$\times 10^6$ D</td>
<td></td>
</tr>
<tr>
<td>DFV</td>
<td>69</td>
<td>10 segments dsRNA (d)</td>
<td>132, 110, 70, 45 (e)</td>
<td>1.37-1.38</td>
</tr>
</tbody>
</table>

(a) Main sources of information: Teninges et al. (1979), Brun and Plus (1980), Gateff et al. (1980), Dobos et al. (1979), Plus and Veyrunes (1978) and Plus et al. (1981)
(b) D = Daltons
(c) Virus also has small projections on the outer surface
(d) Molecular weights not determined
(e) Molecular weight of the smallest polypeptide varies with isolate
suggest that the virus is a contaminant of FCS. However, three lines of evidence are contradictory to this. Firstly, no evidence was found of DXV multiplication in primary cultures of calf kidney cells, chick embryo fibroblasts or in several vertebrate cell culture systems. Secondly, no virus with the properties of this virus has been isolated from mammals, let alone from cattle. Finally, a serologically indistinguishable virus has been isolated from another dipteran host (Culicoides sp.) (Miahle et al., 1983). This virus has been found to have the same biophysical and biochemical properties as DXV. These lines of evidence suggest that DXV is a common contaminant of Drosophila and that some factor in FCS is able to induce the virus to multiply. Although the origin of DXV is still a matter of contention, it will be treated here as a virus of Drosophila.

Dobos et al. (1979) compared the properties of DXV with four other birnaviruses from fish, chickens and molluscs, and found that they were all very similar in their biophysical and biochemical properties. These properties are summarized in Table 1.3.

DFV was originally isolated from French laboratory populations of Drosophila (Plus et al., 1975a). A second strain of the virus has subsequently been isolated from laboratory strains of D. melanogaster from Sweden, and from various permanent cell lines (Brun and Plus, 1980). The two strains of the virus $DFM$ (the French strain) and $DFB$ (the Swedish strain) differ slightly in the sizes of their smallest virion polypeptides (Plus and Brun, 1980; Plus et al., 1981 and see Table 1.3). They do however have identical dsRNA profiles. Unfortunately, although the RNA profiles of a number of isolates of the virus have been described (Plus et al., 1981), the molecular weights have not been given. Therefore, it is not possible to compare the size of the genome/genome segments with those of typical CPVs or reoviruses. The known biophysical and biochemical properties of DFV are summarized in Table 1.3.
1.3.3 Biology and Ecology of Drosophila Viruses.

Many authors have reviewed the available literature on sigma virus and in particular on the biological properties of the virus (see Brun and Plus, 1980; L’Heritier, 1958; Teninges et al., 1980; L’Heritier, 1970). Therefore I will only summarize those features of the biology and ecology of sigma virus that are of direct relevance to this thesis.

Sigma virus may be carried in the gametes of both sexes. In the laboratory the infection may be transmitted by inoculation or transplantation of an infected organ. However it is totally uninfecious by contact, and so in natural populations the only mode of transmission is via the gametes.

Sigma virus infection exists in two states, stabilized and non-stabilized. The ability of males and females to transmit the virus to their offspring is affected by their stabilization state.

Several loci have been identified in D. melanogaster that are responsible for resistance to sigma virus (Ohanessian, 1963; Gay, 1978). Furthermore, genetically distinct strains of sigma virus have been identified in laboratory populations of D. melanogaster (Ohanessian, 1963).

Fleuriet (1976) demonstrated that approximately 50% of natural populations in France were infected with sigma. The frequency of infection within infected populations was generally in the range of 10-20%. One of the resistance alleles, ref(2)P, was found to be polymorphic in French populations (Fleuriet, 1976). Later studies have shown that there is also a polymorphism, for their interaction with the ref(2)P locus, in sigma virus isolates from natural populations (Fleuriet, 1980). This polymorphism is geographically differentiated along with the polymorphism at the ref(2)P locus (Fleuriet, 1986). The geographical differentiation of these two traits suggests that a
mutual adaptation between the virus and the resistance locus has arisen that maintains the virus infection frequency at relatively low levels in natural populations.

On the other hand, population cage experiments demonstrate that the ref(2)P allele has little effect on the frequency of the resistant form of sigma (II), the frequency of infection rising to nearly 100% over the course of time (Fleuriet, 1982). This morph of the virus is the common form in the field populations from which the experimental cage populations were established. The ref(2)P allele did, however, have an effect on the frequency of the sensitive strain of sigma virus (I) which declined in cage populations to levels comparable with those in natural populations. This strain of the virus is not the one commonly encountered in those natural populations that have a high frequency of the ref(2)P allele.

Why and how, then, is the virus maintained at low levels in natural populations? The effect of the virus on certain physiological traits of D. melanogaster e.g. male and female longevity, fertility, sexual selection and egg viability are known to be negligible (Fleuriet, 1981b). The only significant difference is in the lower viability of eggs laid by infected females.

Obviously the mechanisms for maintaining virus frequencies in natural populations are very complex. A variety of environmental effects may put selective pressures on infected flies, but few have been tested under laboratory conditions. One such factor is overwintering. Tests on overwintering (Fleuriet, 1981a) have shown that in some populations the overwintering of adults reduces the infection frequency in the first generations of flies produced in spring relative to those produced in the last generations of the previous autumn.
Table 1.4 Some biological properties of *Drosophila* viruses other than sigma


(b) High = produces death in 3-4 days
Medium = produces death in 7-8 days
Low = may produce death but in > 10 days.

(c) Growth in cell culture is defined as being able to infect cells exogenously or as an endogenous, persistent infection.

(d) C.P.E.; produces a noticeable cytopathic effect after exogenous infection of cell lines

(e) Determined from artificially infected/injected flies

(f) P. Scotti *pers. comm.*
<table>
<thead>
<tr>
<th>Pathogenicity After injection (b)</th>
<th>Growth in cell culture (c)</th>
<th>C.P.E. (d)</th>
<th>Transovarial Transmission</th>
<th>Transmission by contact</th>
<th>Cellular Tropism (e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCV</td>
<td>High</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DAV</td>
<td>Low</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DFV</td>
<td>Low</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Virus G</td>
<td>Low</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>RS virus</td>
<td>Medium</td>
<td>+ (f)</td>
<td>-</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>DXV</td>
<td>Low</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DFV</td>
<td>Low</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

(a) Some biological properties of *Drosophila* viruses other than sigma.

(b) Pathogenicity after injection.

(c) Growth in cell culture.

(d) C.P.E. (cytopathic effect).

(e) Cellular tropism.

(f) + indicates a positive result; - indicates a negative result; ND indicates not determined.
The CO\textsubscript{2} sensitive phenotype of sigma infected flies, and the discovery of various resistance loci has undoubtedly led to it becoming the best understood of the Drosophila viruses in terms of its biology and ecology. Unfortunately, no such simple assay systems are available for the study of the other viruses of Drosophila. Consequently, the study of these viruses, and also of other similar insect viruses, is still very much in its infancy.

Some of the biological properties of the *Drosophila* viruses other than sigma are summarized in Table 1.4.

DCV is by far the most pathogenic of the *Drosophila* viruses. Injection of the virus into adult flies invariably leads to death in 3-4 days, although some strains of the virus have reduced pathogenicity (Plus et al., 1978). In fact some stocks of *D. melanogaster* naturally infected with DCV have an average adult life-span of only eight days (Plus et al., 1975b), less than 20% of normal.

Unlike sigma virus, DCV is not transmitted in the gametes of either sex. Jouisset and Plus (1975) showed that DCV could be transmitted in the following ways:-

(i) adults can become infected after ingesting a high titre of virus. The acquisition of virus in this manner severely reduced the life-span of the infected flies.

(ii) the infection can be passed from artificially (injected) infected flies to uninfected flies by contact.

(iii) the progeny from surface sterilized eggs/dechorionated eggs were not infected by the virus from their infected mothers. Washing of the eggs with saline only, did not stop the virus from being transmitted to the resulting progeny. This implies that the virus is in close association with the chorion of the egg and is transmitted at larval eclosion in some way.
(iv) larvae could be infected per os by contamination of the diet. Transmission in this manner causes a significant decrease in survival through to imago stage and imagos are infected with the virus.

(v) the age of the larvae has an effect on per os infection. First instar larvae were most susceptible to the virus. As the age of the larvae at the time of infection increases the proportion of survivors through to the imago stage increases while the proportion of infected flies decreases.

A particularly interesting consequence of DCV infection was reported by Thomas-Orillard (1984). Females emerging from a persistently infected stock had a significantly greater number of ovarioles than those that emerged from the same stock without the DCV infection. There was also an increase in the fresh weight of the females from the infected line, but they had a shortened developmental time. The increase in the reproductive potential that these results indicate are relatively small, and any advantages gained are likely to be lost at a later stage due to a reduction in adult life-span and reduced hatchability of eggs caused by the virus.

Plus and Golubovsky (1980) found resistance factors against DCV associated with the 1(2)gl lethal. In the fifteen 1(2)gl stocks tested they found variability in susceptibility to DCV ranging from 0-100% mortality after injection of the virus. Unlike the resistance factors to sigma virus, these resistance factors appeared to be acting through the cytoplasm of both parents.

The host range of DCV was tested by Jousset (1976). Of the sixteen species that were tested, DCV was pathogenic, producing 100% mortality in less than 10 days for all of those in the melanogaster subgroup. Species in the subgenus Sophophora - outside the melanogaster subgroup - were also susceptible to DCV, although only 89% of D. ananassae
Figure 1.2 The distribution of *Drosophila* viruses isolated from natural populations of flies

\[ \Delta = DPV \]

\[ \Delta = \text{iota virus} \]

\[ \bullet = DCV \]

\[ \bullet = DAV \]

\[ \star = \text{sigma virus} \]

\[ \ddot{\bullet} = \text{virus G} \]

\[ \dagger = DFV \]

\[ \ddagger = RS \text{ virus} \]

Data from: Plus et al. (1975b), Brun and Plus (1980) and Fleuriet (1986).
Note: "Contact" is the term commonly used in the literature to indicate the interaction between flies that are sharing a common resource, e.g. a culture bottle. The exact mechanism of "contact transmission" is not known but is thought to occur *per os*, by cuticular penetration or by venereal invasion - or combinations of these mechanisms.
individuals were killed in less than 10 days. Members of the subgenus *Drosophila* appeared to be resistant to the virus, except for *D. nasuta* in which 43% of individuals were killed. The virus, did however, multiply in *D. immigrans* without producing symptoms. The drosophilid *Zaprionus tuberculatus* was also not killed by the virus. The pathogenic effect of DCV therefore appears to decrease as the taxonomic distance from *D. melanogaster* increases. It is worth noting however that DCV was pathogenic for the med fly *Ceratitis capitata* (Diptera:Tryphetidae) and was able to multiply in the lepidopteran *Galleria mellonella*.

*Drosophila P* and *A* (DPV and DAV) viruses have very similar biological properties, in fact they were for some time considered to be different serotypes of the same virus (see Jousset and Plus, 1975). Although DPV and DAV are both considered to have low pathogenicity, injection with DPV reduces adult longevity to approximately half that of control injected flies and renders females virtually sterile (David and Plus, 1971).

The modes of transmission of these two viruses are, in many ways, different to those of DCV (Jousset and Plus, 1975) :-

(i) adults can acquire DPV infection after ingesting high titres of the virus,

(ii) transmission of the viruses between naturally infected adults and uninfected adults is a function of the age of the infected flies and the duration of contact. Older flies (>16 days) do not transmit the virus even after prolonged contact,

(iii) injected flies readily transmit the virus to uninfected flies by contact,

(iv) DPV and DAV infected flies, infected by contact, are able to pass the infection on by contact to other flies.
(v) DAV and DPV are both vertically transmitted in the cytoplasm of the egg i.e. dechorionation of the egg does not affect transmission to the progeny from those eggs. Older females (>16 days), however, do not transmit virus in this way, 

(vi) larvae can be readily infected *per os* by contamination of the diet with DPV. Infection with DPV significantly reduces the percentage of emerging flies, and those flies that do emerge are infected with the virus.

DPV and DAV therefore appear to be very closely related biologically, at least in terms of their modes of transmission. DPV has also been found to reduce egg production of naturally infected females. This reduction is more noticeable as females get older (David and Plus, 1971).

Relatively little is known of the biological properties of G virus, RS virus and DFV. What is known is summarized in Table 1.4.

The distribution in natural populations of the picorna-like viruses of *Drosophila* was studied by Plus et al. (1975b). The results of this study along with other data on the distribution of the other *Drosophila* viruses is summarized in Fig. 1.2.

One interesting feature of the distribution of the picorna-like viruses in the above study was that there was a significantly different frequency of infected *D. melanogaster* populations from temperate and warmer countries. None of the strains from France, USA and Canada were infected, however, 11 out of the 21 strains from Morocco, Gabon, French Antilles and the Island of La Reunion were infected. In total, about 40% of all of the populations of *D. melanogaster* tested were found to be infected with one or other of the *Drosophila* viruses. Approximately 30% of these infections were with DCV.
1.4 THE GENUS DROSOPHILA IN AUSTRALIA

1.4.1 Introduction

The family Drosophilidae is comprised of something in excess of 2,500 species (Wheeler, 1981). The 50 or so genera within the family are divided into two subfamilies - Steganinae and Drosophilinae. The genus Drosophila is placed within the Drosophilinae and is the largest genus, containing approximately 60% of the known species.

The genus Drosophila is further subdivided into 15 subgenera (Wheeler, 1981). Many of these subgenera are monotypic or contain only a few members. There are, however, four major subgenera: Drosophila, Hirtodrosophila, Scaptodrosophila and Sophophora. (see Figure 1.3).

Below the taxonomic level of the subgenus, the term species-group is widely used with reference to Drosophila (e.g. see Bock and Wheeler, 1972). The term is used to denote species which share particular combinations of morphological characters and have a presumed common origin. Furthermore, whilst the major subgenera are very much cosmopolitan in their distribution, the species groups are much more restricted in their distributions. The major taxonomic divisions within the Drosophilidae are shown in Figure 1.3.

Each of the major subgenera are represented in Australia. The best represented is the Scaptodrosophila with well over half of the known species. Sophophora and Hirtodrosophila are also well represented, but the subgenus Drosophila is rather depauperate with only 4 endemic species (Bock, 1976) considering that it is by far the largest of the subgenera pandemically. The most striking feature of the Australian fauna then is the dominance of the Scaptodrosophila.

In addition to the endemic fauna, the eight cosmopolitan species (Patterson and Stone, 1952) are also present in Australia. These species, D. melanogaster, D. simulans, D. ananassae, D. immigrans,
Figure 1.3 Taxonomic position and phylogenetic relationships of the Australian Drosophila species discussed in this thesis after Wheeler, 1981; Bock, 1976 and Bock and Wheeler, 1972. Cosmopolitan species (Patterson and Stone, 1952) are marked with an asterisk (*). The number of genera within the Drosophilinae and the number of subgenera within the Drosophila are shown in square brackets.
D. funebris, D. hydei, D. repleta and D. busckii, although not truly world-wide in their distribution, occur in all biogeographical zones. These species are closely associated with the activities of man and are not normally found away from human settlements. Their widespread distribution is therefore thought to have occurred, at least in part, through the agency of man (Dobzhansky, 1965).

1.4.2 Urban Australian Drosophila Communities

Although the endemic Drosophila fauna of Australia is comparatively rich, these species are restricted almost entirely to the rainforests of the eastern seaboard. The introduced, cosmopolitan species on the other hand are restricted almost entirely to urban habitats ("urban" is used to denote any habitat produced by man e.g. rubbish dumps, fruit plantations, gardens or fruit markets).

Most of the viruses isolated from Drosophila have been isolated from D. melanogaster. For this and other reasons, it was decided that this project would concentrate on D. melanogaster populations and the communities in which they occur (see section 1.1). Unfortunately though, little is known of the species compositions of urban Drosophila communities in Australia. In a series of collections made from north Queensland to southern Victoria, Anderson (1985) found that along the eastern seaboard of Australia all collections were composed of one or more of seven species; D. melanogaster, D. simulans, D. ananassae, D. pseudoananassae, D. immigrans, D. sulfurigaster and D. busckii.

D. melanogaster was most common in the northern, warmer regions, D. simulans in the central latitudes with both species being equally abundant in the south. D. ananassae and D. sulfurigaster were found to occur commonly together and were both restricted to localities north of Mackay (21° 9’ S). D. busckii, D. immigrans and D. hydei were all
correlated in their distributions and were most commonly found in the south.

*D. sulfurigaster* is an endemic species of the subgenus *Drosophila* and is common in the rainforests of N. Queensland even though it is also found in urban habitats. *D. ananassae* is considered to be one of the cosmopolitan species, however its presence in Australia may be part of its natural distribution. It is common in the rainforests of S.E. Asia, New Guinea and the western Pacific islands, whilst also occurring commonly in collections from N. Queensland rainforests (see Bock, 1977). A further reason for suggesting *D. ananassae* may have an endemic origin is that *D. melanogaster* has only recently colonised New Guinea (see Bock and Wheeler, 1972) although suitable urban habitats have been available for a long time. Therefore, the presence of *D. ananassae* in New Guinea is presumably not due to the agency of man. Also, the ananassae subgroup has S.E. Asia, the Phillipines, Indonesia and New Guinea as its centre of diversity (see Bock and Wheeler, 1972) and two members of that species group are endemic to Australia, namely *D. pseudoananassae* and *D. bipectinata* (Bock, 1976, 1977).

Bock (1977) found 14 species of *Drosophila* in suburban habitats in Townsville. The most common species in these collections were *D. melanogaster*, *D. ananassae*, *D. pseudoananassae*, *D. bipectinata*, *D. sulfurigaster* and *D. bryani*. *D. simulans* was also collected but was less common than *D. melanogaster*.

The general picture that emerges then of urban *Drosophila* communities is as follows. In northern areas one would generally expect to find *D. melanogaster*, *D. simulans* (less commonly), *D. ananassae/pseudoananassae* and *D. sulfurigaster*, with possibly *D. bryani* and *D. bipectinata*. These populations would therefore be providing points of contact between introduced and endemic species. In the south, however, collections are dominated by the cosmopolitan species *D. melanogaster*, *D. simulans*, *D. immigrans* and *D. busckii*. 
1.4.3 Clinal Variation in Australian *D. melanogaster* populations

One of the reasons that *D. melanogaster* was chosen as the species around which to base this study is that at the populational level it shows marked genetic differentiation. Various genetic parameters have been studied in Australian *D. melanogaster* populations including enzyme gene frequencies, chromosome inversion polymorphisms and P-M hybrid dysgenesis.

A total of eight polymorphic enzyme loci have been studied; Esterase-6 (*Est-6*) and phosphoglucomutase (*Pgm*) (Oakeshott et al., 1981), alcohol dehydrogenase (*Adh*) and glycerol-3-phosphate dehydrogenase (*Gpdh*) (Oakeshott et al., 1982), 6-phosphogluconate dehydrogenase (*Pgd*) and glucose-6-phosphate dehydrogenase (*G6pd*) (Oakeshott et al., 1983a), octanol dehydrogenase (*Odh*) and acid phosphatase (*Acph*) (Oakeshott et al., 1983b).

Allele frequencies at seven out of these eight loci (*Est-6*, *Adh*, *Gpdh*, *Pgd*, *G6pd*, *Odh* and *Acph*) have been found to be significantly associated with distance from the equator. These clines are not restricted to Australian populations of *D. melanogaster*, and the general pattern of allele frequency association with distance from the equator has been found in both hemispheres and in all zoogeographic zones (e.g. see Oakeshott et al., 1981, 1982). In addition similar clines at some of these loci have been found in *D. simulans* populations.

These clines in allele frequency have been considered to be maintained by selective latitudinal gradients, either on the alleles themselves or on closely linked genes. The association between certain climatic variables and allele frequency have therefore been studied. Within Australia there are strong associations between latitude and some of the climatic variables i.e. **T_{Max}** (average daily maximum temperature for the hottest month of the year), **T_{Min}** (average
daily minimum temperature in the coldest month of the year) and $R_{\text{Max}}$ (total rainfall in the wettest month of the year). Less strong associations are found between altitude and $T_{\text{Max}}$ and $T_{\text{Min}}$ (see Oakeshott et al., 1983). However, when the variations in allele frequency were investigated with respect to these climatic variables, the picture that emerges is one of allele frequencies at each locus being associated with a different set of climatic variables. However, in certain cases e.g. $Adh$, the allele frequencies are found to be associated with the same climatic variables in each hemisphere.

Latitudinal clines have also been found for four chromosomal paracentric inversions in natural populations of $D. melanogaster$ (Knibb et al., 1981). These inversions are distributed one each on the four major autosomal arms and are common in $D. melanogaster$ populations the world over. The frequency of each of these inversions increases as the distance from the equator decreases. As with some of the enzyme allele frequency clines, a complementary cline is found for these inversions in the northern hemisphere. Interestingly, Knibb (1983) found that the $Gpdh$ cline could be explained by disequilibrium with the linked inversion $\text{In}(2L)t$, one of the inversions that itself shows a latitudinal cline. However the cline in $Adh$ could not be explained by linkage disequilibrium with any of the common chromosome inversions.

Australian $D. melanogaster$ populations have also been studied for their characteristics in the P-M hybrid dysgenesis system. P-M hybrid dysgenesis is a collection of phenomena that correlate with the insertion and/or deletion of the P transposable element e.g. male recombination and gonadal dysgenesis. Three "population" phenotypes can be scored from the proportion of the dysgenic progeny arising from two reference crosses - cross A (male x reference female) and cross A* (female x reference male). Gonadal dysgenesis in $F_1$ females is the
trait most often scored. The P phenotype produces a high proportion of
dysgenic progeny from cross A and a low proportion from cross A*. The
Q phenotype produces low proportions of dysgenic progeny from both
crosses while the M phenotype produces a high proportion from A* and a
low one from cross A

Boussy and Kidwell (1987) have shown that the three phenotypes are
present in *D. melanogaster* populations from the eastern seaboard of
Australia. Although not truly clinal, there is a latitudinal variation
in the distribution of the phenotypes. Populations from Cairns (16.9°
S) south to Ourimbah (33.4° S) have a P phenotype. Populations from
Cygnet, Tasmania (43.2° S) north to Genoa (37.5S) have the M
phenotype. Populations in between these two zones have the Q
phenotype. Although the P and M phenotypes show reductions in the
severity of the phenotype moving towards the Q phenotype, the
transitions themselves between adjacent phenotype zones appear to be
very sharp.

1.5 SUMMARY

To date, several different viruses have been isolated from various
*Drosophila* species. Most of these viruses have however, been isolated
from *D. melanogaster*. Amongst the viruses isolated from *D.
melanogaster*, the most prevalent type of viruses are the picornalike
viruses, which include DAV, DCV and DPV. Although these are the most
common type of viruses encountered, very little is known of their
distribution in natural populations of *D. melanogaster* and within the
*Drosophila* communities in which *D. melanogaster* occurs.

*D. melanogaster* is cosmopolitan in its distribution and has
undoubtedly been introduced into Australia in the last 200 years by
man. Within Australia it has a widespread distribution and occurs
associated with human settlements down the eastern seaboard of the
continent. At various sites within its distribution it occurs in association with a variety of other Drosophila species thereby, forming communities. The species within these communities are both introduced (cosmopolitan) and endemic. Many of these communities therefore provide natural points of contact between introduced and endemic species of Drosophila. D. melanogaster has also been found to be latitudinally genetically differentiated at the populational level within Australia.

Studies on the viruses found in Australian populations of D. melanogaster therefore offer the opportunity to answer several questions about viruses within and between natural populations/communities of their host(s):

(i) how is a virus distributed within a community? For instance, what are the major host(s) for the virus and has the virus adapted to the endemic species with which D. melanogaster comes into contact?

(ii) is there any geographic variation in the distribution of the viruses, and if so is this related in any way to the known genetic variation in their host?

(iii) what are the possible mechanisms by which the viruses are maintained within populations and how is this related to the ecology of the host?

The approach adopted in this study was to attempt to answer these question as follows: firstly, to isolate and characterise some of the viruses present in natural populations of D. melanogaster. Secondly, to develop sensitive and specific assays for these viruses that would enable detection and identification of the virus in individual Drosophila. The assay system developed would then be applied to assessing the distribution of the viruses within the communities in which D. melanogaster occurs and in different D. melanogaster
populations. Finally, to investigate the effect that the virus(es) have on its host under laboratory conditions and to attempt to elucidate possible mechanisms by which it/they are maintained in natural populations and the effects that the viruses may be having within those populations.

Chapter 2 describes the general virological techniques used throughout this study. The isolation and characterisation of DAV and DCV from laboratory populations of *D. melanogaster* is discussed in Chapter 3, while the cloning of the viral cDNA of these two viruses and the development of a nucleic acid hybridization assay for their detection in single flies is described in Chapter 4. Chapter 5 is concerned with biological characterisation of DAV and DCV, while studies on the effects that the viruses have in persistently infected laboratory stocks are presented in Chapter 6. The data on the distribution of DAV and DCV in natural communities of *Drosophila* are presented in Chapter 7, and Chapter 8 is a general discussion of data contained in the preceding chapters and its relevance to other data on insect viruses.
CHAPTER 2 GENERAL MATERIALS AND METHODS

The materials and methods described in this chapter are those that were used routinely during the course of this study. Other materials and methods more specific to the work included in certain chapters are included in the relevant chapter.

2.1 CHEMICALS AND REAGENTS

Chemical reagents and solvents were supplied by Ajax Chemicals, Sydney, Australia; BDH Chemicals Australia Pty. Ltd., Kilsyth, Victoria, Australia and Sigma Chemicals, St. Louis, USA. Plasticware for cell culture was obtained from Falcon Plastics, Oxnard, California, USA. Schneider’s complete media was supplied by Gibco-Biocult Ltd, Glasgow, Scotland, and foetal calf Serum by Commonwealth Serum Laboratories, Melbourne, Australia.

Agarose for immunological tests and Gel-Bond were obtained from FMC Corporation, Rockland, Ohio, USA.

2.2 SOURCES OF VIRUSES, ANTISERA AND VIRUS-FREE DROSOPHILA STOCKS

Isolates of DCV, DPV, DAV, RS virus, G virus and DF$_3$V were kindly provided by Dr N. Plus (Station de Recherches Cytopathologiques, St.-Christol-lez-Ales, France). These isolates were supplied as groups of 25 Champetieres flies injected with virus immediately before shipment. Upon arrival dead flies were removed and stored at -30°C, surviving flies were harvested ten days after injection and stored at -30°C. CrPV$_{Vic}$ was originally obtained from Dr C. Reinganum (Plant Research Institute, Burnley, Victoria) and had been passaged once through Galleria mellonella by Dr A.J. Gibbs; the resultant cadavers were stored at -30°C. CrPV$_{Bee}$ was a local isolate of the virus from honeybees Apis mellifera provided by Dr D. Anderson (D.S.I.R., Auckland, New Zealand).
D. melanogaster free from known viruses were originally obtained from Dr P. D. Scotti (D.S.I.R., Auckland, New Zealand) and later from Dr N. Plus. Both cultures were derived from the wild-type Champetieres strain (Jousset et al., 1972).

Antisera against DCV \(_z\) (Zagora strain, Plus et al., 1976) DAV, iota virus, DPV, RS virus, G virus and DF\(_{s}V\) (Swedish strain, Plus et al., 1981) were provided by Dr N. Plus. Antisera against CrPV\(_{Vic}\) (Victorian strain, Reinganum et al., 1970), sacbrood virus, black queen cell virus and Kashmir bee virus were provided by Dr D. Anderson.

2.3 LABORATORY CULTURE OF DROSOPHILA

Several species of Drosophila were maintained in the laboratory; D. melanogaster, D. simulans, D. ananassae, D. immigrans, D. sulfurigaster and D. hydei. The artificial diet and conditions used for D. melanogaster were found to be sufficient to maintain all of the species mentioned.

2.3.1 Preparation of Artificial Drosophila Diet

Artificial Drosophila diet was made with the following ingredients:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar</td>
<td>10g</td>
</tr>
<tr>
<td>Bakers Yeast</td>
<td>6g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>12.5g</td>
</tr>
<tr>
<td>Glucose</td>
<td>50g</td>
</tr>
<tr>
<td>Maize Meal</td>
<td>50g</td>
</tr>
<tr>
<td>Raw Wheat Germ</td>
<td>12.5g</td>
</tr>
<tr>
<td>Orthophosphoric acid/propionic acid (14%;4%)</td>
<td>12.5ml</td>
</tr>
<tr>
<td>Water</td>
<td>to 1,000ml</td>
</tr>
</tbody>
</table>

To minimise the risk of the diet becoming contaminated with virus or mites from "stray" flies, fresh diet was always prepared in the
morning before I had been in contact with either Drosophila or Drosophila virus isolates. Bottles were stoppered with cotton wool immediately the diet had set to further reduce the possibilities of the diet being contaminated.

2.3.2 Culturing of Drosophila

All stocks were maintained on artificial diet and kept in an air-conditioned room at 20-22°C. Cultures were transferred to new diet at regular intervals, which varied according to the species being cultured (Table 2.1).

Table 2.1 Developmental times and transfer times used for laboratory culturing of Drosophila at 20-22°C

<table>
<thead>
<tr>
<th>Species</th>
<th>Developmental Times (Days)</th>
<th>Transfer Time (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. melanogaster</td>
<td>12-14</td>
<td>25-28</td>
</tr>
<tr>
<td>D. simulans</td>
<td>14-16</td>
<td>25-28</td>
</tr>
<tr>
<td>D. ananassae</td>
<td>21-25</td>
<td>28-35</td>
</tr>
<tr>
<td>D. sulfurigaster</td>
<td>18-21</td>
<td>28-35</td>
</tr>
<tr>
<td>D. immigrans</td>
<td>28-35</td>
<td>35-40</td>
</tr>
<tr>
<td>D. hydei</td>
<td>21-28</td>
<td>28-35</td>
</tr>
</tbody>
</table>

Occasionally cultures became infested with mites. To remove these mites, adult flies were transferred to empty bottles and aspirated to new diet and subsequently transferred to fresh diet every two days for 10-12 days. Continuous cultures were then re-established from flies treated in this way.

Virus-free flies were maintained in a separate building at 22°C. Culture techniques were as described above, with the exception that
all procedures were carried out first thing in the morning, prior to my having contact with other Drosophila or Drosophila isolates.

2.4 PRODUCTION OF VIRUSES

2.4.1 Propagation of Drosophila Viruses

2.4.1.1 Preparation of Drosophila Virus Inocula

Flies were homogenised in 20 times their own volume of insect saline (0.6% sodium chloride, 0.04% potassium chloride, 0.024% calcium chloride, 0.02% sodium bicarbonate). Homogenates were clarified by centrifugation in an Eppendorf bench-top centrifuge for two minutes. The supernatant was diluted 1:9 with insect saline and sterilised by filtration through a 0.45µm nitrocellulose filter.

2.4.1.2 Propagation of Viruses in Drosophila

Flies were etherised and injected using a modification of the technique of L’Heritier (1952). Micro-needles were pulled on a home-made pulling device from borosilicate glass with an internal diameter of 1.0mm. Each fly received approximately half of its own weight in virus inocula (0.5mg) injected into the abdominal cavity between the second and third ventral tergites. The injected flies were allowed to recover in empty plastic bottles before being transferred onto fresh artificial diet. Control sets of flies were injected at the same time with sterile insect saline.

Injected flies were maintained at 20-22°C and transferred to fresh diet every two days. The dead flies at each transfer were collected and stored at -30°C. Surviving flies were harvested at ten days and stored at -30°C.
2.4.1.3 Propogation of Viruses in Cell Culture

The original isolates of DCV obtained from Dr N. Plus were passed through *Drosophila* cells and purified. This virus was used at a later stages of the research as a reference isolate of DCV (see Chapter 3).

*Drosophila* cell lines (*Drosophila* lines 1 and 2) were kindly supplied by Dr P.D. Scotti. These cells were routinely sub-cultured by the following method.

The cells were grown to confluence in 25 cm\(^2\) plastic culture flasks in Schneider’s complete medium (Schneider, 1964) supplemented with 15% foetal calf serum (FCS). The cells were washed off the bottom of the flask using a sterile pipette and 100µl of the cell suspension was inoculated into 5ml of fresh medium (Schneider’s complete), 15% FCS.

For the passaging of the viruses, a near confluent layer of cells was taken and the culture medium decanted. Fifty microlitres of a sterile isolate of the virus was added to 2ml of medium before it was put onto the monolayer. The virus was allowed to absorb to the cells for 2 hours at 26\(^\circ\)C, the medium removed along with unabsorbed virus and 5 ml of Schneider’s medium, 5% FCS, was added and the cells incubated at 26\(^\circ\)C until a cytopathic effect was visible in most of the cells. Flasks of infected cells were frozen and thawed twice before centrifuging for 10 minutes at 5,000 r.p.m. in an MSE bench-top centrifuge to remove cell debris. The supernatant was frozen at -30\(^\circ\)C until needed and further passages were carried out as detailed above using 100µl of this supernatant to initiate infection of the monolayer.
2.4.2 Propagation of Cricket Paralysis Virus (CrPV)

CrPV was cultured by passaging through *G. mellonella* larvae. Single *G. mellonella* larvae infected with CrPV, *Vic* and CrPV, *Bee* infected honeybee pupae, were homogenised in 1.0ml of insect saline and 0.5ml of chloroform/butanol (1:1), the mixture was vortexed and then spun for five minutes in an Eppendorf bench-top centrifuge. The supernatant was removed and nitrogen was bubbled through it for ten minutes to drive off any excess chloroform/butanol. This extract was then diluted in insect saline (1:9) and sterilized by filtration through a 0.45µm nitrocellulose filter.

*G. mellonella* larvae each received 0.05ml of the sterile isolate injected with a 27 gauge needle between the second and third pair of prolegs. Larvae were placed in batches of 10-15 in sterile plastic petri dishes and kept at 28°C. The petri-dishes were checked every day and the dead larvae removed and stored at -30°C. Larvae surviving until seven days post-injection were harvested and stored at -30°C.

2.5 PURIFICATION OF VIRUSES

2.5.1 Purification of DAV and DCV

The purification procedure for DAV and DCV is summarized in Figure 2.1.

Three to four hundred injected *Drosophila* (collected cadavers and harvested flies) were ground in ten times their own weight of 0.05 M phosphate buffer, pH 7.4. This homogenate was clarified by centrifugation at 10,000 r.p.m. for 10 minutes (Sorvall SS-3 centrifuge). The pellet was discarded and the supernatant was made up to 12.5ml with phosphate buffer and centrifuged for 3 hours at 30,000 r.p.m. (Beckman L2-65B, SW41 rotor, 4°C). The supernatant was discarded and the pellet resuspended overnight in 1.0ml of 0.05 M phosphate buffer, pH 7.4.
300-400 Injected Drosophila

Homogenize in 0.05M phosphate buffer, pH 7.4

Crude homogenate

Centrifuge 10,000 r.p.m., 10 minutes

Supernatant

Centrifuge 30,000 r.p.m., 3 hours

Pellet

Resuspend 0.05M phosphate buffer, pH 7.4

Viral suspension

Centrifuge through 10%, sucrose, 30,000 r.p.m., 3 hours

Pellet

Resuspend 0.05M phosphate buffer, pH 7.4

Viral suspension

Centrifuge through 10-40% (w/v) sucrose gradient, 25,000 r.p.m., 2 hours, and harvest visible bands

Viral suspension

Dilute to 12.5ml and centrifuge 30,000 r.p.m., 3 hours

Pellet

Resuspend 0.05M phosphate buffer, pH 7.4

FINAL PRODUCT
The resuspended pellet was clarified by centrifugation in an Eppendorf bench-top centrifuge and the resultant supernatant was layered onto 10% w/v sucrose (in 0.05M phosphate buffer, pH 7.4) and centrifuged for 3 hours at 30,000 r.p.m. The pellet was drained and resuspended in 1.0ml of 0.05 M phosphate buffer. After clarification in an Eppendorf bench-top centrifuge, the resuspended pellet was layered onto a 10-40% w/v sucrose (in 0.05 M phosphate buffer pH 7.4) and centrifuged for 2 hours at 25,000 r.p.m. (Beckman L2-65B ultracentrifuge, SW41 rotor, 4°C ). Visible bands were collected, diluted to 12.5 ml and centrifuged for 3 hours at 30,000 r.p.m. Standard isolates were produced by resuspending the final pellet in 0.05 M phosphate buffer, pH 7.4, at a ratio of 2.5µl per fly used to initiate the purification.

2.5.2 Purification of CrPV

Thirty infected G. mellonella larvae were ground in a pestle and mortar with 30ml of 0.05 M phosphate buffer, pH 7.4. The homogenate was filtered through four layers of muslin and 15 ml of chloroform/butanol (1:1) was added and the mixture centrifuged at 5,000 r.p.m. for 10 minutes in an MSE bench-top centrifuge. The aqueous phase was removed and nitrogen bubbled through it for 10-20 minutes to drive off excess chloroform/butanol before making the suspension 10% polyethylene glycol 6000 (PEG 6000) and 0.25 M NaCl. This suspension was left at room temperature for 1 hour before centrifugation in a bench-top MSE centrifuge for 5 minutes at 5,000 r.p.m. The pellet was resuspended in 12.5 ml, of 0.05 M phosphate buffer, pH7.4.

Further purification was carried out as described for DAV and DCV (stage 3 through to stage 9 in Figure 2.1). The final pellet was resuspended in 0.05 M phosphate buffer pH 7.4 at a ratio of 30 µl per larvae used to initiate the purification.
2.6 ELECTRON MICROSCOPY

The following technique was routinely used to screen any preparation for the presence of virus(es).

Samples to be checked were mixed 50:50 with 4% w/v ammonium molybdenate. A drop of the mixture was put onto a petri-dish and a carbon-coated formvar covered copper grid was floated on the drop for 5-10 minutes. The grid was removed and dried by touching the reverse side onto hardened filter paper. Samples prepared in this way were examined in a Jeol H500 transmission electron microscope at an accelerating voltage of 75 kV.

2.7 IMMUNOLOGY

2.7.1 Preparation of Antisera

2.7.1.1 Antisera Prepared using Purified Virus

Purified virus particles were diluted 1:1 with 2x insect saline. One third of this suspension was sterilized by filtration through a 45µm nitrocellulose filter and injected into the ear vein of a rabbit. Four weeks later, half of the remaining suspension was mixed 1:1 with Freunds complete adjuvant and injected into the thigh muscle of the same rabbit. After a further two weeks the remaining virus suspension was mixed 1:1 with Freunds complete adjuvant and the rabbit was given another intramuscular injection. After a final four week period the rabbit was bled from an ear vein. The collected blood was allowed to stand at room temperature for an hour before the coagulated red-blood cells were spun down in a bench-top centrifuge (20 minutes at 3,000 r.p.m.). The serum was collected from above the pellet and stored at -30°C until needed. Working dilutions of the antisera were stored at 4°C (diluted in insect saline and made 0.02% w/v sodium azide). All remaining antisera were kept frozen at -30°C until needed.
2.7.1.2 Antisera Prepared Using Gel Precipitates

Antisera were prepared using a modification of the method of Hornitský and Taylor (1983). Approximately 50 precipitin bands from immuno-osmoelectrophoresis gels (see section 2.7.2) were cut out of the agar and washed in several changes of sterile insect saline to remove unprecipitated material. The agar containing the precipitates was homogenized until it was fine enough to pass through a 25 gauge needle. A 24-28 week old rabbit was then given the following sequence of intra-venous injections. On day 0, it received 0.5 ml of the homogenate; on day two, 0.6 ml of the homogenate; on day three, 0.7 ml of the homogenate and so on, with the amount of homogenate being administered increasing by 0.1 ml per day, until the rabbit was receiving 1.0 ml. It was given a further two injections of 1.0 ml spaced three days apart and left for two weeks before bleeding. Isolation of sera from whole blood and storage of the sera were as described in section 2.7.1.1.

2.7.2 Immuno-osmoelectrophoresis

Ten ml of 0.75% agarose in 8.75 mM Tris pH 8.5 was poured onto glass slides (45x75mm). Wells were made opposite one another 1cm apart in the agarose. 20µl of virus sample was loaded into the well nearest to the cathode and 20µl of antisera into the well nearest to the anode. Filter paper wicks soaked in the electrophoresis buffer (35mM Tris pH 8.5) were attached to both ends of the gel and electrophoresis was carried out for 45 minutes at 25 mA/gel at 14-18°C.

2.7.3 Double-Diffusion in Agar

Double diffusion in agar tests were carried out using a modification of the method of Mansi (1958). Five ml of 0.75% agarose was poured onto a strip of GelBond measuring 30x100mm. A hexagonal
array of wells was cut into the agarose spaced 2mm apart from a well in the centre of the array. Antisera was loaded into the central well and the virus samples to be tested into the wells surrounding it. The gel was left overnight in a humid atmosphere for diffusion to take place and precipitin lines to form.

2.7.4 Immunoelectrophoresis

Ten ml of 0.75% agarose in 25mM Tris-HCl, 12.5mM EDTA, pH 7.0, was poured onto glass slides measuring 45x75mm. Wells (2.5mm diameter) were cut in the agarose 1cm apart and 1cm from one end of the slide. The slide was placed into the electrophoresis tank with the wells nearest to the anode and 20µl of virus sample was loaded into each well. Filter paper wicks soaked in electrophoresis buffer (100mM Tris-HCl, 50mM EDTA, pH 7.0) were attached to the ends of the gel. Electrophoresis was carried out at 25mA until a xylene cyanol marker had migrated 45-50mm. Troughs were then cut in the agarose (1x50mm) between the wells and parallel to the direction of migration. These troughs were filled with antisera and the gel left overnight in a humid environment for precipitin lines to form. The relative mobilities of different viruses were calculated with reference to the migration of the xylene cyanol marker.
CHAPTER 3 ISOLATION AND CHARACTERISATION OF DAV AND DCV IN LABORATORY CULTURES OF D. MELANOGASTER

3.1 INTRODUCTION

Before I started this project there was no information available on the viruses that were present in Australian populations of D. melanogaster. Therefore, the first steps in this study were to isolate and characterise the viruses that were to be found in natural populations of D. melanogaster.

In similar studies performed elsewhere (e.g. see Plus et al., 1975b) the standard approach has been to perform successive passages of isolates of wild-caught flies in virus-free D. melanogaster. Generally, it is possible to detect viruses using this method after 2-3 passages (depending on the pathogenicity and growth characteristics of the virus after injection) using a combination of electron microscopic and serological techniques. It was decided to use this technique to assess the viruses present in Australian populations of D. melanogaster.

3.2 MATERIALS AND METHODS

3.2.1 Collection of Drosophila and Establishment of Laboratory Cultures of D. melanogaster

Drosophila were collected from 12 sites within Australia during the period from November 1983 to May 1984 (See section 7.2.1 for more details). Flies were transported to Canberra on artificial diet and sorted into different species upon arrival using the criteria of Bock (1976).

A major problem in establishing cultures of D. melanogaster lies in the difficulty of separating D. melanogaster females from those of
the sibling species *D. simulans*. I used the black posterior band on the sixth abdominal tergite (Gallo, 1973) to discriminate between the two species (95% reliable with Australian *Drosophila* populations; W. Atkinson pers.comm.) and was able to establish mono-specific cultures of *D. melanogaster* (or *D. simulans*) after 3-4 generations of laboratory culture.

Cultures of *D. melanogaster* were established from six localities; Ellis Beach, north Queensland (EB); Cardwell (Mangoes), north Queensland (CaM); Coffs Harbour, north New South Wales (HD); Araluen, south New South Wales (AN); Tamar Valley, Tasmania (TR) and Huonville, Tasmania (HV). Further details of these collection sites are given in Table 7.1.

3.2.2 Self-Titration of Laboratory *D. melanogaster*.

The term "self-titration" is used in this thesis to refer to a procedure I have adopted for propagating viruses from a particular culture of flies. Rather than injecting inocula of a known virus into flies that are free of virus (e.g. see Plus et al., 1975b), an inoculum is made of flies from a given culture and then injected back into flies from the same cohort and the same culture.

3.2.2.1 Ageing of Flies

Eight bottles of flies were kept routinely for each laboratory population of *D. melanogaster*. Adult flies were removed from these bottles 4 days after the culture had been established. The progeny were collected from each bottle every two days, pooled and kept on standard diet until needed. Bottles were discarded after 3-4 weeks, or when new flies ceased emerging. Champetieres flies were aged in the same way.
3.2.2.2 Self-Titration of Aged Flies

50 flies of known age were removed from a culture of flies and a virus isolate made as described in section 2.4.1.1. This isolate was then injected back into flies from the same aged culture from which the flies had been removed to make the isolate. Controls were simultaneously injected with saline. Groups of flies were checked every two days, the dead flies removed, and the surviving flies transferred on to fresh artificial diet. After removal, dead flies were stored at -30°C until needed.

3.2.3 Serology

3.2.3.1 Antisera

DAV purified from Huonville, Coffs Harbour (HD) and Champetteres NZ self-titrations (termed DAV_{HV}, DAV_{HD}, and DAV_{CH} respectively) were used to prepare antisera in rabbits as previously described (section 2.7.1.1.). Antisera directed against DAV (termed anti-DAV_{FR}) and DCV - Zagoz, a strain (anti-DCV_{Z}; Plus et al., 1978) - were those supplied by Dr N. Plus. Antisera against a local strain of CrPV from honeybees Apis mellifera (CrPV_{Bee}) passaged through Galleria mellonella, was produced in rabbits using the same protocol as that for DAV isolates. Antisera was also raised against DAV and DCV using immunooosmoelectrophoresis gel precipitates produced by reacting DAV_{HV} and DCV_{AN} with the appropriate reference antisera provided by Dr. N. Plus (anti-DAV_{FR} and anti-DCV_{Z} respectively). These antisera were termed DAV_{gp} and DCV_{gp} respectively.

A table of the antisera used in this thesis, the virus preparation from which they were produced, and their abbreviations are given in Table 3.1.
Table 3.1 Antisera used in this study and the virus preparations from which they were produced

<table>
<thead>
<tr>
<th>Antisera</th>
<th>Source of Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-DAV&lt;sub&gt;HV&lt;/sub&gt;</td>
<td>DAV produced by self-titration of the Huonville (HV) laboratory stock of D. melanogaster</td>
</tr>
<tr>
<td>anti-DAV&lt;sub&gt;HD&lt;/sub&gt;</td>
<td>DAV produced by self-titration of the Coffs Harbour (HD) laboratory stock of D. melanogaster</td>
</tr>
<tr>
<td>anti-DAV&lt;sub&gt;CH&lt;/sub&gt;</td>
<td>DAV produced by self-titration of the Champetieres&lt;sub&gt;NY&lt;/sub&gt; laboratory stock of D. melanogaster</td>
</tr>
<tr>
<td>anti-DAV&lt;sub&gt;FR&lt;/sub&gt;</td>
<td>DAV produced by propagation of the Fort Lamy isolate from D. melanogaster in Champetieres virus-free stock (Plus et al., 1976). Supplied by Dr N. Plus.</td>
</tr>
<tr>
<td>anti-DAV&lt;sub&gt;gp&lt;/sub&gt;</td>
<td>Gel precipitates formed between DAV from self-titrated Huonville (HV) flies and anti-DAV&lt;sub&gt;FR&lt;/sub&gt; sera.</td>
</tr>
<tr>
<td>anti-DCV&lt;sub&gt;Z&lt;/sub&gt;</td>
<td>DCV produced by propagation of the Zaragoza strain from D. melanogaster in virus-free Champetieres stock (Plus et al., 1978). Supplied by Dr N. Plus</td>
</tr>
<tr>
<td>anti-DCV&lt;sub&gt;gp&lt;/sub&gt;</td>
<td>Gel precipitates formed between DCV from self-titrated Araluen flies and anti-DCV&lt;sub&gt;Z&lt;/sub&gt; sera.</td>
</tr>
<tr>
<td>CrPV&lt;sub&gt;BEE&lt;/sub&gt;</td>
<td>CrPV originally isolated from honeybees and passaged once through larvae of G. mellonella</td>
</tr>
</tbody>
</table>
3.2.3.2 Titration of Antisera

Using standard preparations of CrPV and DAV (see Section 2.5.1, 2.5.2) the end-point dilutions of a two-fold dilution series of each antisera were determined by both immuno-osmoelectrophoresis and double diffusion in agar. The end-point dilution was defined as the lowest dilution at which there was no visible precipitin line formed between virus and antisera. Results are given in Table 3.2.A. Using the antisera at a concentration of two dilutions within the end-point dilution (i.e. for DAVHV antisera the dilution endpoint using immunoelectrophoresis and double-diffusion in agar is 1/64, therefore two dilutions within this endpoint is 1/16), the end-point dilution of a two-fold dilution series of the standard virus preparations was determined by both immunoelectrophoresis and double diffusion in agar (Table 3.2.B).

The end-point dilutions of the antisera tested were approximately the same using either immuno-osmoelectrophoresis or double diffusion in agar. However, the end-point dilutions for the standard virus preparations using immuno-osmoelectrophoresis was 8-16 fold lower than that obtained by double diffusion in agar i.e. 8-16 times more sensitive in detecting the presence of virus. As purified virus was in relatively short supply, all further antisera titrations were performed using immuno-osmoelectrophoresis on a 1/5 dilution of standard virus preparations. Reciprocal dilution end-points for all antisera used in this study are given in table 3.2.C. Antisera were used at a concentration of two dilutions within these end point dilutions. Antisera obtained from other workers were used at the dilutions they suggested (see Table 3.2.C).
Table 3.2 Reciprocal end-point dilutions of antisera used in this study

A. Reciprocal end-point dilutions of anti-CrPV and anti-DAV<sub>H</sub>V sera using immuno-osmoelectrophoresis and double-diffusion in agarose.

B. Reciprocal end-point dilutions of standard preparations of CrPV and DAV<sub>H</sub>V using immuno-osmoelectrophoresis and double-diffusion in agarose.

(1) Standard virus preparation, see section 2.5.1

C. Reciprocal end-point dilutions of all antisera used in this study as measured using immuno-osmoelectrophoresis.

(1) For a list of antisera, and the virus preparations from which they were derived see Table 3.1.

(2) Standard virus preparation, see section 2.5.1

(3) Standard isolate of DCV<sub>0</sub> was obtained by passaging the virus obtained from Dr N. Plus (see Section 2.2) once through Drosophila cells (see Section 2.4.1.3). The supernatant remaining after the passage was spun for 10 minutes at 5,000 r.p.m., the pellet discarded, and made 10% w.r.t. PEG 6000 and 0.25M NaCl. After 1 hour at room temperature the solution was centrifuged at 3,000 r.p.m. for 10 minutes, the supernatant discarded and the pellet resuspended in 0.5ml of 0.05M phosphate buffer, pH 7.4.
Table 3.2 Reciprocal end-point dilutions of antisera used in this study

A.

<table>
<thead>
<tr>
<th>Antisera</th>
<th>METHOD</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Osmoelectrophoresis</td>
</tr>
<tr>
<td>CrPV</td>
<td>128</td>
</tr>
<tr>
<td>DAV_{HV}</td>
<td>128</td>
</tr>
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</table>

B.

<table>
<thead>
<tr>
<th>Virus(1)</th>
<th>METHOD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Osmoelectrophoresis</td>
</tr>
<tr>
<td>CrPV</td>
<td>128</td>
</tr>
<tr>
<td>DAV_{HV}</td>
<td>16</td>
</tr>
</tbody>
</table>

C.

<table>
<thead>
<tr>
<th>ANTISERA(1)</th>
<th>CrPV</th>
<th>DCV_{z}</th>
<th>DCV_{gp}</th>
<th>DAV_{FR}</th>
<th>DAV_{HV}</th>
<th>DAV_{HD}</th>
<th>DAV_{CH}</th>
<th>DAV_{gp}</th>
</tr>
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<tbody>
<tr>
<td>CrPV</td>
<td>32</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DCV_{AN}</td>
<td>16</td>
<td>128</td>
<td>16</td>
<td>0</td>
<td>256</td>
<td>128</td>
<td>64</td>
<td>0</td>
</tr>
<tr>
<td>DCV_{O}</td>
<td>16</td>
<td>256</td>
<td>16</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>DAV_{HV}</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>256</td>
<td>128</td>
<td>64</td>
<td>64</td>
<td>16</td>
</tr>
</tbody>
</table>
3.3. RESULTS

3.3.1 Passaging of Viruses in Champetieres\textsubscript{NZ} Flies

Isolates of DAV, DPV, RS virus, G virus and DF\textsubscript{5}V obtained from Dr N. Plus were passaged in 0-2 day old Champetieres\textsubscript{NZ} flies. After 10 days the surviving flies were harvested, ground in ten times their own volume of 0.05M phosphate buffer, pH7.4, and subjected to immuno-osmoelectrophoresis against the relevant antisera. None of these crude extracts formed precipitin lines with the antisera. Furthermore, none of the injected isolates gave rise to a mortality above the level in the saline injected controls (RS virus should cause mortality in six-eight days after injection; N. Plus, pers.comm.).

Electron microscopic examination of all of the crude extracts from the passaging of these viruses, revealed the presence of small isometric particles only, approximately 30nm in diameter. The concentration of these virus particles was estimated to be much less than that normally found when extracts from single G. mellonella larvae infected with CrPV ground in 1ml of 0.05m phosphate buffer, pH7.4, were examined in the electron microscope. Even after the virus-particles in these crude preparations were concentrated by centrifugation in an air-fuge, it was not possible to discern any particles with a morphology different from those already observed. This was not to be expected if the injected viruses had replicated because at least two of them (DFV and RS virus) have very different morphology from the particles that were observed in the electron microscope. The observation of the same type of particle in all of these preparations therefore suggested that there was a virus present in the Champetieres\textsubscript{NZ} flies that was in some way inhibiting the growth of the injected viruses.
3.3.2 Self-Titration of Laboratory D. melanogaster

Passaging of Drosophila viruses through the Champetieres, NZ flies suggested that there was actually an endogenous virus in this stock. This immediately presented a problem with respect to screening isolates from natural populations of D. melanogaster, as it could no longer be assumed that the Champetieres, NZ flies were virus-free. Thus the origin of any virus produced by screening, using this reference stock, would be uncertain. Furthermore, the virus present in the Champetieres, NZ flies also seemed to inhibit the growth of any other viruses introduced into the system. To circumvent the problem of introducing "foreign" viruses into the flies being injected, it was decided to screen laboratory populations of D. melanogaster for endogenous viruses by the self-titration method, this method relied on injecting an inocula of the viruses that may already be present within a group of flies back into representatives from that same group of flies.

Self-titurations were performed on 300 flies from the laboratory stocks established from populations collected at Ellis Beach, Cardwell (Mangoes), Coffs Harbour (HD), Araluen, Tamar Valley, Huonville and Champetieres, NZ. Saline controls were injected simultaneously for each stock of flies and each paired group of flies - self-titrated and controls - were checked every two days for dead flies. Dead flies were removed and stored at -30°C. The experiment was terminated ten days after the flies had been injected, and all surviving flies were frozen at -30°C.

In general, two types of mortality-time response curves were observed (Figure 3.1), which I have designated type A and type B. In stocks in which a type A curve was observed there is very little difference in mortality over time between the self-titrated flies and the saline controls. In contrast, in stocks in which a type B curve is
Figure 3.1 Mortality time response curves for self-titrated (o--o) and saline injected (x--x) Huonville (HV), Coffs Harbour (HD), Araluen (AN) and Ellis Beach (EB) laboratory stocks.

**TYPE A MORTALITY RESPONSE CURVES**

![Type A Mortality Response Curves](image)

**TYPE B MORTALITY TIME RESPONSE CURVES**

![Type B Mortality Time Response Curves](image)
observed, the mortality in the self-titrated flies is much greater than that of the saline-injected controls. Crude extracts of flies that had died during the course of the experiment, or of those that had survived until the tenth day were made by grinding flies in ten times their own volume of 0.05M phosphate buffer, pH 7.4. These extracts were then examined by electron microscopy and subjected to immuno-osmoelectrophoresis against reference antisera.

The crude isolates were found to contain small icosahedral particles as observed in the isolates obtained by passaging viruses in ChampetieresNZ flies. These viruses were estimated to be at a higher concentration than previously observed. Immuno-osmoelectrophoresis revealed that extracts from flies exhibiting type A mortality-time response curves produced precipitin lines with anti-DAVFR sera, while those from type B produced precipitin lines with anti-DCVZ sera. Each preparation reacted with only one of these two antisera, and none of them reacted with any of the other reference antisera. Extracts prepared from Cardwell (Mangoes), Coffs Harbour, Tamar, Huonville and ChampetieresNZ self-titrated flies reacted with only anti-DAVFR sera, while those prepared from Ellis Beach and Araluen self-titrated flies reacted with only anti-DCVZ sera. None of the preparations reacted with any of the other reference antisera.

The mortality-time response curves and the serological results demonstrate that each of the laboratory cultures established from natural Australian populations of D. melanogaster, along with the ChampetieresNZ stock, were persistently infected with one of two viruses, DAV or DCV. Each laboratory stock appeared to be infected with only one of the two viruses. To further characterise the viruses present in these laboratory stocks it was decided to prepare antisera against purified virus preparations.
3.3.3 Serological Characterisation of DAV and DCV from Laboratory Stocks

Virus was purified (as described in section 2.5.1) from self-titrated flies taken from the laboratory stocks Huonville, Coffs Harbour (HD) and ChampetieresNZ. These virus preparations were found to react with only anti-DAVFR serum when tested by immuno-osmoelectrophoresis and so were termed DAVHV, DAVHD and DAVCH respectively. These virus preparations were then used to produce antisera as described in section 2.7.1.1. The antisera produced from these virus preparations are termed anti-DAVHV, anti-DAVHD and anti-DAVCH respectively (Table 3.1).

Although the purified virus preparations reacted with only anti-DAVFR sera, the antisera raised against DAVHV, DAVHD and ChampetieresNZ were found to react not only with standard preparations of DAV but also with DCV (see Table 3.2C). This implied that either the DAV purified from the laboratory stocks is serologically related to DCV, or that DCV was a low-level contaminant of the virus preparations which was undetectable by immuno-osmoelectrophoresis.

Double diffusion in agar was carried out to test the serological relationship between DAV and DCV isolated from the laboratory stocks of D. melanogaster and also to ascertain the origin of the specificity for both DAV and DCV exhibited by anti-DAVHV, anti-DAVHD and anti-DAVCH sera. The reaction between DAV and DCV was found to be one of non-identity (Figure 3.2B). This result is in agreement with their original assignment to different serogroups (Plus et al., 1975). Furthermore, a reaction of non-identity was observed when any of the antisera prepared from Huonville, Coffs Harbour or ChampetieresNZ virus preparations was used on its own, and also when a mixture of anti-DAVFR and anti-DCVZ was used (see Table 3.2). The results of these tests suggest that there were in fact two distinct viruses.
FIGURE 3.2

A. Double diffusion in agar showing the reaction of partial identity between CrPV and DCV₀ and DCVₑᵦₑ

Rₐ = anti-CrPVₑᵦₑ serum (see Table 3.1)
O = DCV₀
R = CrPVₑᵦₑ
E = DCVₑᵦₑ

B. Double diffusion in agar showing the reaction of non-identity between DAVHV and DCVₑᵦₑ. Note that there are reactions of identity between DAVHV and DAVHD and between DCVₑᵦₑ and DCV₀.

Hₐ = anti-DAVHV serum (see Table 3.1)
O = DCV₀
E = DCVₑᵦₑ
H = DAVHV
D = DAVHD

C. Immunoelectrophoresis (see Section 2.7.4) of DAVHV (H) and DCVₑᵦₑ (E), showing reactions against anti-DAVgp (Aa), anti-DAVHV (Ha) and anti-DCVgp (Ca). See Table 3.1 for derivation of antisera. Migration of the xylene cyanol marker is indicated(→)
present in the laboratory populations and that the dual specificity of the anti-DAV sera produced in this laboratory was due to DCV being a minor contaminant of the DAV preparations.

DCV and CrPV have been shown to be serologically related (Reinganum and Scotti, 1972), and to exhibit a reaction of partial identity (Plus et al., 1978). There are however two important features of the relationship between DCV and CrPV:

(i) all isolates of DCV react with anti-CrPV sera, and show a reaction of partial identity with CrPV, but,

(ii) while all isolates of DCV show reactions of identity when reacted against DCV antisera, CrPV will react only with antisera produced from some isolates of DCV i.e. will react with anti-DCV₀ sera but not with anti-DCV₂ (Plus et al., 1978).

Using preparations of DCV purified from self-titrated flies from the laboratory stocks Ellis Beach and Araluen (DCV_{EB} and DCV_{AN} respectively), a reaction of identity was observed with DCV₀ in double-diffusion in agar tests (see Figure 3.2A and Table 3.3). With all isolates of DCV tested (DCV_{AN}, DCV_{EB} and DCV₀) a reaction of partial identity was observed with CrPV when anti-CrPV sera was used (Figure 3.2A and Table 3.3). However, CrPV did not react with either anti-DCV₂ sera or with the antisera produced from gel-precipitates of DCV_{AN} and anti-DCV₂ sera (this antisera is termed anti-DCV₂gp - see Section 3.2.3.1 and Table 3.1 and 3.3). These data demonstrate that the DCV preparations isolated from laboratory stocks of D. melanogaster are serologically indistinguishable from DCV₀ when reacted with anti-DCV₂ sera. However, as has previously been observed for some isolates of DCV (i.e. DCV₂; Plus et al., 1978), although DCV_{EB} and DCV_{AN} showed a reaction of partial identity with CrPV when anti-CrPV sera was used, the antisera raised against the DCV_{AN} isolate did not react with CrPV (c.f. Plus et al., 1978).
Results of all double-diffusion in agar tests are summarized in Table 3.3.

A difference in electrophoretic mobility was also noted between DCV and DAV (Figure 3.2C)

3.4 DISCUSSION

As information was not available at the start of this project on the viruses that were to be found in Australian populations of Drosophila, the successive passages technique (e.g. see Plus et al., 1975b) was used to isolate and characterise the viruses that were present in natural populations of D. melanogaster.

Initial attempts to passage stocks of virus obtained from overseas suggested that the reference virus-free stock of D. melanogaster I was using was, in fact, contaminated by a small icosahedral virus. The presence of this virus appeared to be inhibiting the replication of viruses injected into these flies. To circumvent this problem the technique of self-titration was developed.

Using this technique, viruses of serotypes A and C were found to be ubiquitous in laboratory stocks of D. melanogaster derived from wild-caught Australian flies. There are two possible routes by which the viruses could have entered these stocks: either they were present in the wild caught flies used to initiate the stocks, or they were contaminants that had entered the stocks after their establishment in the laboratory. The six laboratory stocks tested (Ellis Beach, Cardwell (Mangoes), Coffs Harbour, Araluen, Huonville and Tamar Valley) had all been established in the laboratory for at least 18 months before they were screened for endogenous viruses. During this time, they had never been exposed to either DAV or DAV infected flies. This observation strongly suggests that the DAV infection in these stocks derived from the viruses' presence in the natural populations used to establish the laboratory cultures.
Table 3.3 Serological characterisation of the viruses and antisera used in the current study (a)

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Reference Virus (b)</th>
<th>DAVHV</th>
<th>DAVHD</th>
<th>DCVAN</th>
<th>DCVEB</th>
<th>DCV0</th>
<th>CrPV_BEE</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAVHV</td>
<td>DAVHV</td>
<td>*</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>DAVHD</td>
<td>DAVHD</td>
<td></td>
<td>*</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DAVCH</td>
<td>DAVCH</td>
<td></td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DAVGP</td>
<td>DAVHV</td>
<td>*</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DAVFR</td>
<td>DAVHV (c)</td>
<td>*/+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DCVGP</td>
<td>DCVAN</td>
<td>-</td>
<td>-</td>
<td>*</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>DCVZ</td>
<td>DCV0 (d)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>*/+</td>
<td>-</td>
</tr>
<tr>
<td>CrPV_BEE</td>
<td>CrPV_BEE</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)/+</td>
<td>*</td>
</tr>
</tbody>
</table>

+ = Reaction of identity
(+)= Reaction of partial-identity
0 = Reaction of non-identity
- = no reaction
* = homologous reaction
ND = Reaction not performed

(a) List of antisera is to be found in Table 3.1

(b) Reference virus is the virus from which the antisera was prepared except for:
   (c) No isolate of DAV used to produce DAVFR (i.e Fort-Lamy strain, Plus et al., 1976) was available
   (d) Isolates of DCVZ were not available, however, DCV0 and DCVZ show a reaction of identity with anti-DCVZ (Plus et al., 1978)
   (e) Reinganum and Scotti (1976) showed a reaction of identity between CrPV and DCV with CrPV antisera
Although certain precautions were followed in the handling of flies e.g. diet preparation (see section 2.3.1), to reduce the risk of contamination of laboratory cultures, DCV was being used by myself - in a separate laboratory - during the time between the establishment of the laboratory cultures and the isolation from them of DCV. So, although it seemed probable that the DAV in these cultures was derived from the flies from which they were established, it was possible that DCV was a laboratory contaminant in these stocks.

The method of self-titration proved useful in assessing the viruses that were present in laboratory populations of *D. melanogaster*. A qualitative assessment of the viruses that may be present in natural populations can also be made, based on their presence in laboratory stocks. However, any quantitative estimates of virus distribution based on this method cannot be made, due to the possibility that simultaneously maintained cultures in close contact could be subject to cross-contamination. Nonetheless, it seems reasonable to suppose from the data presented here that DAV was present in at least one of the natural populations of *D. melanogaster* which were used to establish the laboratory cultures.

DAV and DCV isolated from laboratory populations proved to be serologically related to DAV and DCV previously isolated from *Drosophila* (Plus et al., 1975; Jousset et al., 1972). Although no reference isolates of DAV were available for comparison with DAVs isolated from the Cardwell (Mangoes), Coffs Harbour, Huonville and Tamar Valley laboratory stocks, their reactions with anti-DAV_{FR} sera, their non-identity with DCV, and inability to react with all other reference antisera show that they belong to the DAV sero-group (Plus et al., 1975b). DCV prepared from the Araluen and Ellis Beach laboratory stocks showed two features consistent with a close serological relationship with other isolates of DCV (Plus et al.,
1978). Firstly, their reactions of identity with DCV₀ using either DCV₂ or DCV₂EB antisera demonstrated that they were members of the DCV sero-group. Secondly, their reactions of partial identity with CrPV demonstrated that within the DCV sero-group they are more closely related to other isolates of DCV than they are to CrPV.

Interestingly, the failure of all antisera produced in this study that have titres against DCV to react with CrPV, is similar to previous reports for antisera produced against some, but not all, isolates of DCV (Plus et al., 1978). Plus et al. (1978) found that of two antisera prepared against different isolates of DCV, only that against DCVₓ (a French isolate) reacted with CrPV in gel-diffusion in agar. Under normal conditions the second antiserum (anti-DCVₓ - the reference anti-DCV sera used in the present study) would not react with CrPV; only by increasing the antigen concentration a hundred-fold and using immuno-osmoelectrophoresis could a reaction be produced. So, although all isolates of DCV tested to date have been found to be serologically indistinguishable in their reaction with anti-DCV sera, two putative groups of DCV can be proposed on the ability of antisera raised against viruses from the two groups, to react with CrPV. One group would contain all of those viruses in which antisera produced against them reacts with CrPV (this group would contain DCVₓ). The second group would include those isolates to which antisera produced against them does not react with CrPV. This second group would contain DCV₂ and also all of the isolates of DCV that have been isolated in this study from laboratory populations of D. melanogaster established from Australian wild-caught flies.

Although self-titration of D. melanogaster stocks appeared to produce only a single virus as judged by immuno-osmoelectrophoresis, antisera raised against these virus preparations (anti-DAVₓ₁, anti-DAVₓ₂ and anti-DAVₓ₃) reacted with both DAV and DCV. There are two
possible explanations for this anomaly. Firstly, DCV might be a minor contaminant of the DAV virus preparations that is undetectable by immuno-osmoelectrophoresis. Secondly, the structural proteins of DAV and DCV might share antigenic determinants. The second of these possibilities can be rejected primarily because of the reaction of non-identity between DCV and DAV using the antisera produced from self-titrated virus preparations (see Figure 3.2B). Furthermore, when DAV antisera was produced using gel-precipitates, the antisera did not react with DCV. DCV must therefore have been a minor contaminant of the virus preparations produced by self-titration of laboratory stocks. As self-titration relies only on injecting into a stock viruses that are present within that stock, DCV must have been present in the laboratory stocks along with DAV.

In conclusion, two small icosahedral viruses—DCV and DAV—were identified in laboratory populations of D. melanogaster established from wild caught flies. The viruses were serologically related to those isolated overseas. The data described here suggest that the DAV was derived from the wild-caught flies used to establish the laboratory cultures. Further studies on the distribution of these viruses in natural populations of Drosophila and the possible effects of the viruses on the biology of the flies were dependent on the development of sensitive and specific assays for the viruses. The criteria decided upon for the selection of an appropriate assay system and its development will be described in the following chapter.
CHAPTER 4 CLONING AND CHARACTERISATION OF FRAGMENTS OF THE DAV AND DCV GENOMES

4.1 INTRODUCTION

The results presented in the previous chapter strongly suggested that DAV and DCV were the viruses most likely to be encountered in wild-caught populations of Drosophila. In order to make a detailed survey of the distribution of these two viruses within natural populations it was first necessary to develop sensitive and specific detection techniques for both viruses. The criterion for sensitivity that I decided upon was that the technique should be able to detect virus particles in a single fly with greater sensitivity than the most insensitive serological techniques i.e. double-diffusion in agar or immuno-osmoelectrophoresis. Therefore, there was a choice of three types of technique: biological, serological or biochemical.

Biological detection techniques involve bioassay in either whole animals or in cell culture. Both bioassays have the advantage that they are sensitive, since they rely on virus multiplication. Although relatively non-specific, in combination with serological techniques such as virus neutralization, they do have the power to become relatively specific (e.g. see Scotti, 1983). However, for whole-animal bioassay of Drosophila viruses, it is essential to be sure that a Drosophila stock is virus-free in order to avoid the types of problems presented in the previous chapter (see Section 3.3.1.). Maintaining and regularly screening such a virus-free stock to ensure that it remains virus-free is a major technical problem requiring specialized facilities that are unavailable in Canberra. Moreover, the technique is inappropriate for screening large numbers of individual Drosophila to gain information on infection frequencies within populations - the technique would require each isolate to be passaged at least two or
three times before viruses would be detectable by either electron microscopy or serology. Assaying for viruses in cell culture has many of the problems encountered in the whole-animal bioassay. Many Drosophila cell lines have been found to harbour endogenous viruses (Plus, 1978) and screening of individual flies would require at least two or three passages of each isolate before the viruses would be detected.

So, although potentially very sensitive, the biological assay of Drosophila viruses at the populational level would prove to be very expensive both in terms of time and materials. In addition to these difficulties, other viruses in the detection system being used would be likely to complicate matters and probably obscure the presence of the viruses that are being screened for. For these reasons, it was decided that an in vitro detection system would be preferable for DAV and DCV.

There are a large number of serological detection methods available for the detection of viruses. They range in sensitivity, for small RNA viruses, from being able to detect 10,000 ng virus/ml using double diffusion in agar (based on an end-point for this method of $10^{12}$ particles/ml for small icosahedral viruses; Gibbs and Harrison, 1976) to detecting approximately 2 ng/ml using enzyme linked immunosorbant assay (ELISA) (Anderson, 1984). In his comparative study of several immunological techniques for detecting honeybee viruses, Anderson (1984) found that ELISA was as sensitive as radioimmunoassay (RIA) and that both methods were in the region of a thousand times more sensitive than the next most sensitive serological techniques, immuno-osmoelectrophoresis and immune serum electron microscopy (ISEM). ELISA has the advantage over RIA in that background levels were found to be much lower at very dilute virus concentrations.
As no data were available on the amount of virus that might be expected to be found in an individual *D. melanogaster*, it was assumed that the most sensitive technique available for detecting DCV and DAV should be preferred.

ELISA appeared to be the most sensitive serological technique available. Initial tests with CrPV using the indirect F(ab')\(_2\) coating antibody technique (see Anderson, 1984) gave detection levels of 10-50 ng/ml. Assuming that similar detection levels could be reached for DCV and DAV, and that individual flies were to be ground in 200 µl of buffer, this would be equivalent to 2-10 ng of virus/fly (5-25 x 10\(^7\) virus particles). However, when the same technique was applied to the detection of DAV and DCV using DAV\(_{HV}\) antisera, these detection levels could not be repeated using purified virus preparations. Furthermore, considerable problems were encountered with high backgrounds arising from the presence of host material. For these reasons, and also because of difficulties in obtaining mono-specific antisera against either of the viruses, it was decided to develop a biochemical technique for the detection of these viruses.

Nucleic acid hybridization appeared to offer the sensitivity and the specificity required for this study, but it has not, so far, been applied to the detection of any small insect RNA virus. However, in several studies on plant viruses there have been reports of the use of nucleic acid hybridization in the detection of viruses in their insect vectors. Boulton et al. (1984) using cDNA prepared directly from the ssDNA of maize streak virus (MSV) were able to detect as little as 0.0125ng of virus in the presence of insect extracts and could readily detect the virus in leafhoppers allowed to feed on MSV infected plants. Although it would be desirable to apply this method to the detection of DAV and DCV i.e. by synthesising cDNA probes directly from the viral RNAs, there are many practical limitations.
Not the least of these limitations is the large amount of viral RNA required. Furthermore, this RNA would have to be prepared from purified virus produced by the self-titration method, which has already been shown to be dogged by the production of mixed virus preparations. For these reasons, cloning a cDNA copy of the viral RNA seemed to be the most sensible approach to take. Even though mixed virus preparations could present problems when using this approach, once a cDNA copy of a fragment of the viral genome has been cloned, large amounts of that fragment are available for characterisation to ascertain from which virus they are derived.

Cloning of viral cDNA has been used successfully in the development of cDNA probes for small RNA viruses of plants e.g. beet western yellows virus (BWYV) (Skotnicki et al., 1987) and barley yellow dwarf virus (BYDV) (Waterhouse et al., 1986). Probes produced from viral cDNA clones of these viruses have proved capable of detecting between 0.5 ng (Waterhouse et al., 1986) and 1 ng (Skotnicki et al., 1987) of virus. Furthermore, it has also proven possible to detect BWYV in its aphid vector using probes prepared from viral cDNA clones.

In summary, the bioassay systems available for the present study, undoubtedly contained endogenous viruses. Hence, it was not possible to use them for screening populations which contained unknown viruses. Moreover, these bioassay systems are impracticable for populational studies on Drosophila viruses. Therefore, a biological detection system for DAV and DCV was rejected. The difficulty of producing monospecific antisera against DAV and DCV mitigated against the use of a serological detection system. In addition, the most sensitive serological technique available, ELISA, was found to be inapplicable for screening Drosophila due the high background produced by the insect material. Although the problem of mixed virus preparations was still to be overcome, it seemed that cloning of viral cDNA was the best practical solution to the development of an assay system.
This chapter describes the cloning of cDNA synthesised from DAV and DCV RNAs and the development of a viral cDNA hybridization assay system that is capable of detecting DAV and DCV in isolates from individual Drosophila.

4.2 MATERIALS AND METHODS

4.2.1 Materials

4.2.1.1 Chemicals and Reagents

Chemical reagents and solvents were supplied by Ajax Chemicals, Sydney, Australia; BDH Chemicals Australia Pty. Ltd., Kilsyth, Victoria, Australia and Sigma Chemicals, St. Louis, USA.

Nucleotides (dATP, dCTP, dGTP and dTTP) were obtained from Boehringer, Mannheim, W. Germany. α^{32}P-labelled nucleotides were supplied by Amersham, UK.

Components of bacterial culture medias were obtained from Difco Laboratories, Detroit, USA.

4.2.1.2 Enzymes

Restriction endonucleases and other enzymes used were supplied by the following companies; BRESA, Adelaide, Australia; Boehringer; Pharmacia, Uppsala, Sweden and New England Biolabs, Beverley, USA.

Some enzymes were also purified by Anne MacKenzie at the Research School of Biological Sciences, ANU, Canberra.
4.2.1.3 Bacterial Strains and Media

Bacterial strains RRL and JM101 were stored in 50% glycerol at -20°C.

The composition of media used for bacterial culture were as follows:

**LBG**

- Yeast extract: 5g
- Bacto-tryptone: 10g
- NaCl: 5g
- Glucose: 5g
- Distilled water: to 1000ml

**YT**

- Bacto-tryptone: 15g
- Yeast extract: 5g
- NaCl: 5g
- Distilled water: to 1000ml

**M9 Minimal Salts**

- \( \text{Na}_2\text{HPO}_4 \): 6.0g
- \( \text{KH}_2\text{PO}_4 \): 3.0g
- NaCl: 0.5g
- \( \text{NH}_4\text{Cl} \): 1.0g
- Distilled water: to 990 ml

After autoclaving, 10ml of a 0.01M solution of CaCl\(_2\) is added.

Solid media contained 1.5% w/v agarose. Ampicillin was added to a final concentration of 40µg/ml.
4.2.1.4 Vectors

Purified pBR322 plasmid DNA was a gift from Anne McKenzie, and the ds replicative form of M13mp18 was provided by Dr Paul Keese.

4.2.2 Preparation of Nucleic Acid

4.2.2.1 Viral RNA

DAV and DCV from 400 Huonville and Araluen self-titrated flies respectively, were purified as previously described (Section 2.5.1). Each virus preparation was checked for the presence of DAV and DCV by immuno-osmoelectrophoresis. Concentrated virus preparations from the Huonville and Araluen flies reacted against only anti-DAV and anti-DCV sera respectively, and were therefore considered to consist of only a single virus. At the final stage of the purification procedure the viruses were resuspended in 2 ml of RNA extraction buffer (0.01M Tris-HCl pH 7.4, 0.01M KCl, 1.5mM MgCl₂, 0.2% w/v SDS). Viral RNA was further purified by the method of Both and Air (1979). Briefly, proteinase K was added to the virus suspension to a final concentration of 125µg/ml and incubated at 56°C for 20 minutes. NaCl was added to a concentration of 0.15M and 3.0 ml of water-saturated phenol was added and mixed into the suspension by shaking at 56°C for 5 minutes. Three ml of chloroform was then added and the mixture was vortexed occasionally at room temperature for 5 minutes. The aqueous phase was separated by centrifugation at 5,000 r.p.m. for 10 minutes before being removed and made 0.3M sodium acetate. Two volumes of cold ethanol were then added to this suspension and the RNA was precipitated by centrifugation at 10,000 r.p.m. for 15 minutes. Two further ethanol precipitations were performed before the RNA was resuspended in 0.3M sodium acetate. RNA stocks were kept at -20°C under two volumes of ethanol.
4.2.2.2 Supercoiled Plasmid DNA

Large quantities of supercoiled plasmid DNA were isolated and purified on CsCl gradients (Maniatis et al., 1982).

4.2.2.3 Bacteriophage DNA

Single-stranded DNA from recombinant M13 phage was purified by a modification of the method of Messing et al. (1977). Briefly, JM101 cells were grown overnight in M9 minimal media. This culture was diluted 1/100 with 2x YT media and divided into 2ml aliquots. A single plaque was picked from a culture plate with a toothpick and transferred to one of these aliquots and incubated at 37°C for 5-7 hours. Cells were precipitated by centrifugation and the supernatant made 2.5% PEG (8000) and 0.25M NaCl. This mixture was left at room temperature for 45 minutes before the phage was pelleted by centrifugation for 5 minutes at 4°C. The pellet was resuspended in 100µl of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and 50µl of phenol saturated with TE was added. Phage DNA was extracted and precipitated by the addition of sodium acetate, pH 5.5, to a final concentration of 0.3M, and two volumes of cold ethanol. The DNA was washed with 70% ethanol and dried in vacuo before resuspension in TE buffer.

4.2.3 Restriction Enzyme Digestion of DNA

Single restriction enzyme digests of dsDNA were carried out in accordance with the manufacturers directions. Enzyme was added at a ratio of 2 units/µg of DNA. Digestions with more than one enzyme were usually made in TA buffer (33mM Tris-acetate pH7.9, 66mM potassium acetate, 10mM magnesium acetate, 0.5mM DTT and 100µg/ml BSA). Reactions were stopped by the addition of a half volume of loading dye (30% w/v sucrose, 0.09% w/v bromophenol blue, 50mM EDTA)
4.2.4 Electrophoresis of DNA

4.2.4.1 Agarose Gel Electrophoresis

Electrophoresis of DNA was performed in horizontal 1% agarose gels prepared in TAE buffer (0.04M Tris-acetate, pH7.8, 0.001M EDTA). Electrophoresis was generally carried out at 10-25V/cm for 3-4 hours at room temperature. DNA was visualized by staining the gel with ethidium bromide (1.5µg/ml) for 10 minutes before exposing to short-wave (254nm) ultra-violet light.

4.2.4.2 Polyacrylamide Gel Electrophoresis.

Non-denaturing vertical slab gels were prepared by dilution of a 50% polyacrylamide solution (24:1 acrylamide: bis-acrylamide) to the required concentration using 2x TBE buffer (1x TBE = 0.089M Tris-borate, 0.089M boric acid, 0.002M EDTA, pH 8.0).

Polymerization of the acrylamide was achieved by adding ammonium persulphate to a final concentration of 0.1% and N,N,N',N' tetramethylethylenediamine (TEMED) to a concentration of 0.01%.

For denaturing gels, solid urea was added to give a final concentration of 7M.

Prior to electrophoresis, DNA samples were mixed 50:50 with dye. For non-denaturing gels, the dye was composed of 30% sucrose, 10mM EDTA, 0.02% bromophenol blue and 0.02% xylene cyanol; samples were loaded directly after addition of the dye. For denaturing gels, the dye consisted of 95% deionized formamide, 10mM EDTA, 0.02% bromophenol blue and 0.02% xylene cyanol; after addition of the dye the samples were boiled for 2 minutes before loading.

Electrophoresis of DNA was carried out at between 500 and 1300V (approximately 30V/cm) in 2x TBE buffer, until the bromophenol blue had migrated to the bottom of the gel.
4.2.5 Molecular Cloning Protocols

A summary of the cloning protocols used in this study are given in Figure 4.1.

4.2.5.1 cDNA Synthesis

A. Random Priming (RP) Method

Approximately 1µg of RNA was primed with 0.1µg herring sperm random primers and 0.1µg of oligo dT\(^{(12-18)}\). cDNA synthesis was carried out in 50mM Tris-HCl pH 8.3, 4mM dithiothreitol (DTT), 6.5mM magnesium acetate and 1mM dATP, dCTP, dGTP, dTTP (dN buffer) using AMV reverse transcriptase. The reaction was extracted twice with equal volumes of phenol and chloroform and the cDNA/RNA hybrid precipitated twice by the addition of sodium acetate, pH 5.5, to a final concentration of 0.3M, and two volumes of cold ethanol. RNA/DNA hybrids were resuspended in second-strand buffer (50mM Tris-HCl pH 7.4, 50mM magnesium chloride, 10mM potassium chloride, 10mM ammonium sulphate, 0.2mM DTT, 20µg/ml BSA and 0.2mM dATP, dCTP, dGTP, dTTP) and the RNA strand was replaced using RNase H and DNA polymerase I to form dsDNA (Gubler and Hoffman, 1983).

The dsDNA was extracted twice with an equal volume of phenol and chloroform and precipitated twice with 0.3M sodium acetate and two volumes of cold ethanol before drying and resuspension in dN buffer. The overlapping ends of the dsDNA were filled-in using the Klenow fragment of DNA polymerase I to generate blunt-ended fragments.

BamHI linkers were ligated to the blunt-ended DNA using \(T_4\) DNA ligase and \(T_4\) RNA ligase (see Section 4.2.5.3). Concatamerised linkers were removed by digestion overnight in TA buffer with BamHI. The DNA was extracted twice with an equal volume of phenol and chloroform and high molecular weight DNA precipitated immediately by centrifugation in an Eppendorf bench-top centrifuge for 10 minutes at 4°C after the
Figure 4.1 Summary of cloning protocols used in the cloning of cDNA fragments of the RNA genomes of DAV and DCV

1. Prime with Poly(T)
2. Prime with random primers and Poly(T)
3. Reverse transcribe to produce RNA-DNA hybrid
4. Remove RNA (RNase H) and synthesise second DNA strand
5. Restrict with 3 enzymes that have 4bp recognition sequences to generate blunt-ended ds DNA
6. Fill in overlapping ends to generate blunt-ended ds DNA
7. Ligate Bam HI linkers onto ds DNA molecules
8. Ligate into the Sma I site of M13
9. Ligate into the Bam HI site of pBR322
10. Select Recombinants containing virally related cDNA
addition of sodium acetate, pH 5.5 to a final concentration of 0.3M, and two volumes of cold ethanol.

B. Oligo-d(T) Priming Method

To generate clones specifically from the 3’ end of the viral genome, viral RNA was primed with poly(T) only, rather than with random primers and poly(T) as described above.

Approximately 0.5µg of viral RNA was primed with 0.01µg of oligo d-T(12-18). cDNA was synthesised using AMV reverse transcriptase in the presence of 20mM Tris-HCl pH8.3, 20mM KCl, 10mM DTT, 3mM magnesium chloride, 6µg/ml BSA and 2mM dATP, dCTP, dGTP, dTTP. The RNA/DNA hybrid was extracted twice with an equal volume of phenol and chloroform and precipitated twice with sodium acetate, pH 5.5 to a final concentration of 0.3M, and two volumes of cold ethanol. Precipitated DNA was dried and resuspended in second-strand buffer. RNA was replaced with DNA using DNA polymerase I, RNase H and T₄ DNA ligase. The dsDNA was extracted twice with an equal volume of phenol and chloroform and precipitated with sodium acetate, pH 5.5, to a final concentration of 0.3M and two volumes of cold ethanol. Precipitated DNA was dried and resuspended in sterile distilled water.

The DNA was divided into three aliquots and digested using three different restriction enzymes with four base recognition sites as described in section 4.2.3. The digested DNA was extracted twice with an equal volume of phenol and chloroform and precipitated with sodium acetate, pH 5.5, to a final concentration of 0.3M, and two volumes of cold ethanol. The DNA was washed with 70% ethanol and dried in vacuo.

To further remove any impurities before cloning the DNA was precipitated with spermine. Two µl of 100mM spermine was added to the DNA which was left on ice for 15 minutes. DNA was precipitated by centrifugation in an Eppendorf centrifuge at 4°C for 10 minutes. The
supernatant was removed and the pellet washed with 70% ethanol before addition of 150µl 75% ethanol v/v, 0.3M sodium acetate, pH 5.5, and 0.01M magnesium acetate. The pellet was left on ice for an hour, the supernatant removed, and the pellet washed with 70% ethanol and dried in vacuo. The final pellet was resuspended in 10mM Tris-HCl pH 7.4, 10mM magnesium chloride.

4.2.5.2 Preparation of Vectors

Vector DNA was digested with restriction enzymes as described in section 4.2.3. The DNA was extracted twice with an equal volume of phenol and chloroform and precipitated with sodium acetate, pH 5.5 to a final concentration of 0.3M, and two volumes of cold ethanol.

To reduce self-ligation of vectors, the 5' terminal phosphates of the digested DNA were removed using calf-intestinal alkaline phosphatase (CAP). DNA (approximately 10µg) was treated with 2 units of CAP in the presence of 100mM Tris base pH 10.8, 0.2% SDS at 37°C for 30 minutes. After this time had elapsed, a further two units of CAP was added and the reaction resumed for another 30 minutes. The dephosphorylated DNA was extracted twice with an equal volume of phenol and chloroform, and precipitated with sodium acetate, pH 5.5, to a final concentration of 0.3M and two volumes of cold ethanol.

4.2.5.3 Ligation Conditions and Subcloning From pBR322 into M13

DNA was ligated into the appropriately digested dephosphorylated vector at a ratio of 1:10 in the presence of 66mM tris-HCl, pH 7.8, 10mM magnesium chloride, 10mM DTT and 1 mM ATP using T₄ DNA ligase. Reactions were generally carried out at room temperature for 6 hours. However, for blunt-ended ligations the reaction was allowed to proceed overnight at 4°C.
Virally related inserts were removed from pBR322 by digestion with BamHI and ligated into the BamHI site of M13 under the conditions described above.

Note, clones in pBR322 will be referred to with the abbreviation p e.g. pAB16; whereas clones in M13 will have the prefix m. So, M13 clones derived from the subcloning of DNA from the pBR322 clone will retain the identification code but will have the prefix "m".

4.2.5.4 Transformation Procedures

A. Transformation with pBR322

Competent RRI cells were prepared in the following way. RRI cells were inoculated into 40ml LBG media and incubated at 37°C until the cell suspension reached an optical density at 640nm of 0.4 to 0.6. The cells were sedimented by centrifugation at 5,000 r.p.m. for 5 minutes (SS34 rotor) and resuspended in 2ml of 50mM CaCl₂. The cell suspension was kept on ice until ready for use.

Ligation mixes containing pBR322 were added to 200µl of competent cell suspension and incubated at 4°C for 30 minutes. The cells were then heat-shocked at 42°C for 1.5 minutes. The transformation mix was then inoculated into 3ml of LBG media and incubated at 37°C for 1.5 hours. 150µl aliquots of this culture were plated onto LBG plates containing ampicillin and incubated overnight at 37°C.

B. Transformation with M13

Competent JM101 cells were prepared in the following way. Cells were grown in 40ml 2xYT media until they reached an optical density at 640nm of 0.4-0.6. Cells were sedimented by centrifugation at 3,000 r.p.m. for 5 minutes (SS34 rotor) and resuspended in 10ml TFB buffer (10mM potassium methyl sulphoxide, pH 6.2, 100 KCl, 45mM MnCl₂, 10mM CaCl₂ and 3mM hydroxy amine cobalt chloride). The cells were left on
ice for 5 minutes and then sedimented as described above. Sedimented cells were resuspended in 3.5ml TFB buffer and incubated on ice for 5 minutes before the addition of 130µl of deionised dimethyl formamide (DMFO). The cell suspension was incubated on ice for a further 5 minutes and then 7µl of β-mercaptoethanol was added. After a further 5 minute incubation on ice 130µl of DMFO was added. Cells were kept on ice until ready for transformation.

Ligation mixtures involving M13 were added to competent JM101 cells and the cell suspension heat-shocked at 37°C for 1.5 minutes. Heat-shocked cells were then transferred into soft agar (0.7%) containing 2x YT (1.6% w/v tryptone, 1% w/v yeast extract, 0.5% NaCl), 65mM isopropyl-thiogalactoside (IPTG) and 0.013% 5-bromo-4-chloro-indolyl-β-D-galactoside (X-Gal) and plated onto 1.5% agar.

4.2.5.5 Selection of Recombinants

A. Recombinants in pBR322

Recombinants in pBR322 were selected by the following method. Ampicillin resistant colonies were replated onto LBG media containing ampicillin. Plasmid DNA was isolated from these colonies using a modification of the method of Holmes and Quigley (1981). Briefly, cells were removed from the culture plate and transferred to 200µl of STET buffer (8% w/v sucrose, 0.05M EDTA, 0.05M Tris-HCl, pH 8.0, 5% Triton X-100). Ten µl of freshly prepared lysozyme (10mg/ml) solution were added, the mixture vortexed and then boiled for 1 minute. Cell debris and chromosomal DNA were removed by centrifugation in an Eppendorf bench-top centrifuge for 10 minutes. Plasmid DNA was precipitated from the supernatant by the addition of an equal volume of isopropanol and incubation at -20°C for 15 minutes. DNA was recovered by centrifugation for 5 minutes in an Eppendorf centrifuge, washed with 70% ethanol and dried in vacuo. Each pellet was resuspended in 15µl of sterile distilled water.
Purified plasmid DNA was subjected to electrophoresis in 1% agarose as described in section 4.2.4.1. Plasmid DNAs visibly larger than pBR322 were selected for further characterisation.

B. Recombinants in M13

Insertion into the poly-linker region of M13 invariably leads to the disruption of the reading frame of the β galactosidase gene, and hence the bacteria containing recombinant bacteriophage are unable to produce an active gene product. β-galactosidase will normally catalyse the conversion of the X-Gal in the plating medium to produce a blue product. Therefore, colonies infected with recombinant bacteriophage give rise to colourless, rather than blue, turbid plaques because of their lack of active β-galactosidase.

4.2.6 Hybridization Protocols

4.2.6.1 Synthesis of $^{32}$P-Labelled Hybridization Probes

Three types of $^{32}$P-labelled probes were made in the course of this study. The first of these was cDNA synthesised after the priming of viral RNA with random primers and/or poly(T); this will be referred to as a "cDNA probe". The second type of probe was made from denatured dsDNA primed with random primers and will be referred to as a "ds DNA probe". The final type of probe was synthesised from clones in M13, and will be termed a "strand-specific probe".

cDNA probes were synthesised from viral RNA after priming with either 12-18mer random-primers prepared from herring sperm DNA or with oligo dT(12-18). Reaction conditions were as described in section 4.2.5.1A, but dATP was replaced with $^{32}$P-dATP. RNA was hydrolysed from the RNA/DNA hybrid by incubation at 65°C for 1 hour in the presence of 15mM NaOH. $^{32}$P-labelled cDNA was separated from unincorporated nucleotides by chromatography on a Sephadex-G50 column or by electrophoresis on a 4% polyacrylamide gel.
For the preparation of ds DNA probes, denatured linear dsDNA was primed with 12-18mer random-primers prepared from herring sperm DNA. Synthesis of $^{32}P$-labelled DNA was carried out in the presence of 10mM Tris-HCl, pH 7.4, 1mM magnesium chloride, 1mM dGTP, dCTP, dTTP and $^{32}P$-dATP using the Klenow fragment of DNA polymerase I. The reaction was stopped by the addition of EDTA to a final concentration of 5mM. $^{32}P$-labelled cDNA was separated from unincorporated nucleotides by Sephadex-G50 column chromatography.

Strand-specific probes were synthesised in the following way. Single-stranded DNA from recombinant phage was primed with the ml3 17mer sequencing primer at a ratio of 1µg:1ng. Transcription across the virally related insert was carried out in 10mM Tris-HCl pH7.4, 1mM magnesium chloride in the presence of 0.5mM dGTP, dTTP and $\alpha^{32}P$-dATP and $\alpha^{32}P$-dCTP using the Klenow fragment of DNA polymerase I. After DNA synthesis the dsDNA was digested with restriction endonucleases to remove the virally related insert (or the required fragment of the insert). The insert was then separated from the phage DNA by electrophoresis on a non-denaturing 4% polyacrylamide gel. The $^{32}P$-labelled insert was then excised from the gel and the DNA eluted overnight in 1%SDS, 10M Tris-HCl pH7.4, 1mM EDTA.

4.2.6.2 Transfer of Nucleic Acids to Hybridization Membranes

A. Southern Blot Transfers

DNA was transferred from agarose gels to nitrocellulose by a modification of the method of Southern (1975). The DNA was denatured by soaking the gel for 30 minutes in 0.5M NaOH, and subsequently neutralised by washing for 15 minutes in 1.5M NaCl, 0.5M Tris-HCl, pH 7.4. Denatured DNA was then transferred to nitrocellulose by blotting the gel overnight in a 20x-2x SSC gradient. Nitrocellulose filters were baked for 2-4 hours at 80°C before hybridization.
B. Colony Filter Transfers

pBR322 transformed colonies were transferred directly to circular nitrocellulose filters. After transfer the colonies were lysed by treatment of the filters for 5 minutes with 0.5M NaOH. The filters were then treated for 5 minutes with 1M Tris-HCl, pH 7.9, and for 5 minutes with 0.5M Tris-HCl, pH 7.4, 1.5M NaCl. After drying the filters were baked for 2-4 hours at 80°C before hybridizations.

M13 containing turbid plaques were transferred to Biodyne nylon transfer membranes (Pall Ultrafine Filtration Corp., USA) after plates had been kept at 4°C for 1 hour to stop adhesion of the soft agar overlay. Filters were treated as described above.

C. Dot-Blotting

For dot-blotting of purified RNA and DNA, 1µl of the sample was spotted onto Hybond-N hybridization membrane (Amersham, UK) and allowed to dry. Nucleic acids were bound to the membrane by baking at 80°C for 4-6 hours.

Vacuum dot-blotting was performed using a 96 well standard-design dot-blotting apparatus. Hybond-N was pre-treated by soaking in 12x SSC. A support of 3 thicknesses of blotting paper was placed under the Hybond-N to reduce lateral diffusion of the samples being applied. Samples dot-blotted in this way were allowed to air dry before baking at 80°C for 4-6 hours.

D. Transfer of Whole Virus from Agarose to Hybond-N

Whole virus was subjected to electrophoresis as described in section 2.7.4. After electrophoresis, the agarose gel was removed from the glass plate and soaked in 2x SSC for five minutes. Whole virus was then transferred directly to Hybond-N as described in section 4.2.6.2A. Filters were baked at 80°C for 4-6 hours.
4.2.6.3 Hybridization Conditions

A. DNA-DNA Hybridizations

DNA-DNA hybridizations were carried out in buffer containing:

- **SSC** 6x
- **EDTA** 0.01M
- Denhardt’s solution* 5x
- **SDS** 0.5%
- Denatured salmon sperm DNA 100µg/ml

* 1x Denhardt's solution = 0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin.

Filters to be hybridized were pre-hybridized overnight at 65°C in hybridization buffer. Pre-hybridization buffer was removed and replaced with fresh hybridization buffer. All ³²P-labelled probes were boiled for 5 minutes before adding to the hybridization solution. Hybridization was allowed to proceed overnight at 65°C.

After hybridization the filters were washed twice in 2x SSC (45°C) and once in 0.1x SSC, 0.1% SDS (45°C). Each wash was for approximately 45 minutes. After washing, filters were air dried before autoradiography.

B DNA-RNA Hybridizations

DNA-RNA hybridizations were carried out in the following buffer:

- Deionised formamide 40% v/v
- **SDS** 1%
- **SSC** 3x
- Denhardt’s solution 5x
- Tris-HCl, pH 8.0 10mM
- **EDTA** 1mM

Hybridization conditions were as described for DNA-DNA hybridization except that all operations were carried out at 45°C.
4.2.7 DNA Sequencing

M13 cDNA clones were subjected to the T-reaction of the dideoxynucleotide sequencing method of Sanger et al. (1977). The reaction was stopped by the addition of dye and the cDNA subjected to electrophoresis on a 6% denaturing polyacrylamide gel (Section 4.2.4.2).

4.3 RESULTS

4.3.1. Identification and Characterisation of Cloned Fragments of the DCV and DAV Genomes

4.3.1.1 Generation and Selection of Clones

Fragments of the genomes of DAV and DCV were initially cloned by the random-priming method (Section 4.2.5.1A) into the BamHI site of pBR322. This cloning was relatively inefficient, producing only 584 and 653 ampicillin resistant colonies respectively.

Approximately 200 colonies were selected from the DAV cloning, plasmid DNA isolated (Section 4.2.5.5A), and sized by agarose gel electrophoresis. The plasmid DNAs visibly larger than pBR322 were transferred to nitrocellulose and probed with cDNA probes made from DAV viral RNA using random primers. Those clones that hybridized strongly to this probe were selected for further characterisation.

This selection procedure yielded seven clones; pAA60, pAB6, pAB16, pAB31, pAB32, pAB35 and pAB46. Virally related inserts were removed from these clones and sized by agarose gel electrophoresis. The plasmids contained inserts of sizes approximately 300bp, 360bp, 720bp, 360bp, 350bp, 480bp and 360bp respectively.

Colonies produced from the cloning of DCV were screened for plasmids containing virally related inserts by colony filter hybridization. Four clones were selected in this way for further characterisation: pCK2, pCK3, pCK28, pCK32. These plasmids contained inserts of approximately 1100bp, 660bp, 490bp and 460bp respectively.
Further characterisation of all clones was performed by digesting each plasmid with the restriction endonucleases BamHI, EcoRI, HindIII, PstI, PvuII, EcoRV, NdeI, ClaI, SacI, BglII, ApaI, XhoI and SmaI. Cross-hybridization studies showed that the DAV clones comprise two overlapping groups. The first group is composed of pAA60, pAB6, pAB16, pAB31, pAB32 and the second group of pAB35 and pAB46.

All four of the DCV clones were found to be overlapping by cross-hybridization.

Fragments from the DCV genome were also cloned into M13 using the oligo-d(T) method. Double-stranded DNA was digested with AluI, HaeIII or RsaI, and ligated into the SmaI site of M13mp18. This cloning procedure yielded in the region of 700 turbid plaques (containing presumed recombinant phage with virally related inserts). However, the relative efficiency of cloning the fragments generated by the three restriction endonucleases AluI, HaeIII and RsaI were very different, with a ratio of approximately 10:1:1 white plaques respectively.

The turbid plaques generated by this procedure were screened using the plaque filter transfer method (Section 4.2.6.2B). \(^{32}\)P-labelled cDNA was synthesised from DCV viral RNA primed with oligo-d(T)\(_{12-18}\) (Section 4.2.6.1) and fractionated into two size classes by polyacrylamide gel electrophoresis; this produced a 300-800 base class and one of >800 bases. All plaques were probed independently with both cDNA probes.

Two definite classes of colonies could be identified by the intensity with which they hybridised to these cDNA probes - weak and strong. It is possible, using the colony lift-off method, that turbid plaques that hybridize weakly are infected with phage that is carrying the strand of DNA complementary to the viral RNA, and that the hybridization is in fact with the replicative (ds) form of the phage - which is at very low copy number relative to that of the ss phage.
The proportion of plaques hybridizing to the 300-800 base cDNA were, including both strongly and weakly hybridizing colonies, 67%, 38% and 20% for the AluI, Rsai and HaeIII generated clones respectively. Proportionately more plaques hybridized to the large cDNA (>800 bases) than to the smaller cDNA i.e. 70%, 52% and 25%. The cloning of the Rsai fragments also produced some blue plaques that hybridized to the small cDNA. These were presumably due to the cloned insert being "leaky" with respect to disrupting the β-galactosidase gene, and hence the bacteria is able to produce an active gene product.

After probing with the large cDNA, M13 from 30 turbid plaques from AluI and Rsai generated clones, which hybridized strongly to this probe, were selected and purified for further characterisation.

The small cDNA was also found to hybridize to the two DCV clones pCK3 and pCK28. This suggested that these two clones were located within 800 bases of the 3' end of the viral RNA.

The 30 purified clones from each of the AluI and Rsai clones were screened using the small (300-800 base) cDNA probe from DCV RNA and strand-specific probe from mCK3. Of the 30 AluI clones, 21 hybridized to the small (300-800 base) cDNA class. As all 30 hybridized with the large cDNA class, this means that only 9 of these clones came from regions greater than 800 bases 5' to the poly(A) tail. Furthermore, only 4 of these 30 clones did not hybridize with the strand-specific probe made from mCK3 (CK3 lies between 500-1100 bases 5' to the poly(A) tail, see Figure 4.2). With the Rsai generated clones, 12 of the 30 hybridized with the small cDNA class. However, only 2 of the clones did not hybridize to the strand-specific probe from mCK3.

These results, with the AluI and Rsai generated clones, show that the cloning procedure used was most efficient in cloning fragments towards the 3' end of the viral RNA genome.
Several AluI and RsaI clones that hybridized with the small cDNA and mCK3 strand-specific probe were T-tracked. T-tracking involves carrying out only the T reaction from the dideoxynucleotide sequencing method on an M13 clone. This is then electrophoresed as per normal sequencing protocol. From the characteristic pattern produced by the vector (M13) DNA on the 5' side of the polylinker region (in which any potential insert will be located), it can be ascertained if there is an insert, its approximate size and whether it contains a fragment of the viral poly(A) tail. From T-tracking results, one M13 clone, mCA10, was found that contained a fragment of the viral poly(A) tail and had a relatively large insert. Strand-specific probe made from mCA10 hybridized to both pCK3 and pCK28 and also to DCV RNA. It did not however, hybridize with any other pBR322 clones from either DAV or DCV, or with DAV RNA. A BamHI site was found in the dsDNA produced by synthesising DNA across the insert of mCA10. Cross-hybridization studies show that this site corresponds to the BamHI site at the 3' end of pCK28. This site is presumably virally encoded and is not derived from the BamHI linkers used in the cloning of pCK28. The arrangement of the DCV clones relative to one another and to the viral RNA is shown in Figure 4.2.

The physical map of DAV clones is shown in Figure 4.3.

4.3.1.2 Specificity of Cloned cDNA

One problem encountered during the characterisation of the clones from DAV and DCV was the presence of contaminating DAV RNA in the DCV RNA preparations. In a control experiment, in which reciprocal hybridizations were performed between cDNA probes prepared from the two viral RNAs and the cloned cDNAs from the opposite virus, DCV cDNA probes hybridized to pAA60, pAB6, pAB16 and pAB32. Hybridization stringency tests using a strand-specific probe made from mAB16 showed
Figure 4.2 Physical map of DCV clones showing cDNA restriction endonuclease sites.

Sites were not found for the restriction endonucleases: ApaI, ClaI, EcoRV, NdeI, SacI, SmaI and XhoI.
Figure 4.3 Physical map of DAV clones showing cDNA restriction
endonuclease sites.

Sites were not found for the restriction endonucleases; ApaI, ClaI, SacI, SmaI and XhoI.

Note: (i) The positions of the two groups of overlapping clones, relative to the viral RNA, are not known.
(ii) pAB6 and pAB31 contain identical inserts. Only pAB6 is illustrated here.
that the relative hybridization between the probe and the DCV RNA did not vary at temperatures between 35°C and 65°C (Figure 4.4A). Even after washing at 65°C (0.1% SDS, 0.1x SSC), the relative hybridization did not change (Figure 4.4B). These results strongly suggest that DAV RNA was a contaminant of DCV RNA preparations.

DCV and DAV, in whole virus form, can be separated by electrophoresis in agarose (see Figure 3.2C). After electrophoresis, the whole virus, and hence the viral RNA, was transferred to Hybond-N and the membrane probed with cDNA against DCV RNA, and strand-specific probes from mAB16 and mCK2 (Figure 4.4C). The results showed that the strand-specific probe from mAB16 was only able to detect DAV RNA, while the probe from mCK2 was only able to detect DCV RNA. cDNA prepared from DCV RNA was able to detect both DAV and DCV. These data demonstrate that, although the DCV RNA preparation contains both DCV and DAV RNA, the clones generated from it are specific for DCV while those generated from the DAV RNA preparation are specific for DAV.

The relationship of DAV and DCV to CrPV was also tested using hybridization (Figure 4.4A and 4.5A). Strand-specific probes from M13 clones of both DAV and DCV did not hybridize to CrPV RNA. Low stringency washing did not increase the amount of detectable hybridization to CrPV RNA of any of these probes.

### 4.3.2 Selection of Clones For Use as Probes

All of the M13 cDNA clones derived from the sub-cloning of inserts from pBR322 were tested for their ability to hybridize to purified DAV and DCV. All of the clones proved to be specific against only the virus from whose RNA they were cloned.

It was found that using two cDNA clones (or fragments of them) as hybridization probes from any one virus increased the sensitivity of detection of viral RNA above that of using either of the two fragments.
A. Hybridization stringency tests. Samples of DCV (C), CrPV (R) and DAV (A) RNA were spotted directly onto Hybond-N. Each column on the filter carries a five-fold dilution series of each RNA comprising 30ng (top), 6ng, 1.4ng, 0.28ng and 0.056ng (bottom - visible only in a). After blotting all filters were probed with strand-specific probe (see Section 4.2.6.1) synthesised from mAB16 and hybridized at 35°C (a), 45°C (b) and 65°C (c). All post hybridization washes were carried out at 45°C as described in section 4.2.6.3B.

B. RNA samples as described in A were spotted directly onto Hybond-N and hybridization was carried out as described above. Post hybridization washes were performed at 65°C in 0.1x SSC, 0.1% SDS.

NOTE: the relative hybridization of the probe does not vary with the hybridization temperature (A), or the temperature of the post-hybridization wash (B), suggesting that DAV RNA is a contaminant of the DCV RNA preparation.

C. Whole virus transfer to Hybond-N (see Section 4.2.6.2D). Virus samples were electrophoresed as described in Section 2.7.4, and transferred to Hybond-N (see Section 4.2.6.2D).

Virus Samples: E = DCVEB, O = DCVO, H = DAVH, D = DAVHD, N = DCVAN - containing both DAV and DCV (see Table 6.1)

The point to which the xylene cyanol marker had migrated is marked (---), as are the wells into which the virus samples were loaded (●). Filters were probed with:

a - strand-specific probe synthesised from mCK2
b - strand-specific probe synthesised from mAB16
c - cDNA synthesised from random-primed DCV RNA.

NOTE: DAV and DCV have different electrophoretic mobilities (see Figure 3.2C), DCV migrates to just above the xylene cyanol and DAV to just below. The two strand-specific probes detect only the virus from which they were cloned. In contrast, the cDNA from DCV RNA detects both DAV and DCV, demonstrating that the DCV RNA is contaminated with DAV RNA.
alone. For this reason, two strand specific probes from cloned fragments of each virus were routinely used for all hybridizations:

(i) DAV probing: the large BamHI-PstI fragment from mAB16 and the insert from mAB35.

(ii) DCV probing: the large BamHI-PstI fragment from mCK2 and the insert from mCK3

The BamHI-PstI fragments from mAB16 and mCK2 were used in preference to the whole insert because their size is more amenable to elution from 4% polyacrylamide.

It is also worth noting here, that although the two fragments used for the probing of DCV have a region of overlap (see Figure 4.2), this region is relatively small and did not appear to interfere with the detection sensitivity.

4.3.3 Detection Sensitivity of Purified RNA and Viruses

By spotting RNA samples directly onto Hybond-N, it was possible to reliably detect between 0.48ng and 0.24ng of material from either DCV or DAV (Figure 4.5A and B).

The O.D.\textsubscript{260} of purified samples of DAV and DCV was taken to be an approximate measurement of the RNA concentration in the whole-virus sample (1 O.D.\textsubscript{260} = 40µg/ml RNA). Using this approximation, and dotting whole virus samples directly onto Hybond-N, between 0.240ng and 1.2ng of DAV RNA and between 1.2ng and 6ng of DCV RNA could be detected (Figure 4.5A and B). The difference in hybridization sensitivity between the purified RNAs and the RNA in nucleoprotein form has been noted before for a structurally similar plant virus (Waterhouse et al., 1986). This difference probably reflects the RNA in nucleoprotein form being less available for hybridization.

The detection sensitivity for purified virus was tested further by taking three separate samples of both DAV and DCV, measuring their
FIGURE 4.5

Samples of whole virus (V), viral RNA (R) and CrPV RNA (C) spotted directly onto Hybond-N (see Section 4.3.3).

A. DCV and DCV RNA; the filter was probed with strand-specific probes specific for DCV (see Section 4.3.2). Hybridization and post-hybridization treatment of the filters was as described in Section 4.2.6.3B.

B. DAV and DAV RNA; the filter was probed with strand-specific probes specific for DAV (see Section 4.3.2). Hybridization and post-hybridization treatment of the filter was as described in Section 4.2.6.3B.

NOTE: the amount of RNA in the whole virus samples was estimated to be equivalent to that of the viral RNA samples (see Section 4.3.3). Each column on the filter carries a five-fold dilution series of the appropriate sample (whole virus or viral RNA) containing 30ng (top), 6ng, 1.2ng, 0.24ng, 0.048ng and 0.0096ng (bottom - not detectable on either filter) of RNA.

The dilution end-points for each virus are:

<table>
<thead>
<tr>
<th>Virus</th>
<th>Sample</th>
<th>END-POINT</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCV</td>
<td>Whole Virus</td>
<td>1.20ng</td>
</tr>
<tr>
<td></td>
<td>Viral RNA</td>
<td>0.048ng</td>
</tr>
<tr>
<td>DAV</td>
<td>Whole Virus</td>
<td>0.24ng</td>
</tr>
<tr>
<td></td>
<td>Viral RNA</td>
<td>0.048ng</td>
</tr>
</tbody>
</table>
O.D.260 and finding their hybridization dilution end-point. Using the O.D.260 as an approximation to the RNA concentration in the samples, the detection sensitivities were found to be 0.23ng, 0.35ng and 0.4ng for DAV, while for DCV they were found to be 0.46ng, 0.55ng and 2.6ng. The immunological dilution end-point was also measured for each sample by immuno-osmoelectrophoresis. It was found that the ratios of hybridization : immunological end-points (hybridization dilution end-point/immunological dilution end-point) were 0.5, 0.25 and 0.5 for DAV whereas for DCV they were 2, 4 and 1. If the immunological end-point is a fair reflection of the amount of virus particles present, i.e. there is no variation in the proportion of "empty" particles in the DAV and DCV samples (which would effectively decrease the immunological end-point dilution disproportionately to the amount of RNA present), then this result suggests that the RNA from DCV, while in the nucleoprotein form, is less readily available for hybridization than is the DAV RNA.

Finally, it has been shown that immuno-osmoelectrophoresis is approximately 8-16 times more sensitive than double-diffusion in agar (see section 3.1.3.2). Taking the end-point of double-diffusion in agar to represent a concentration of \(10^{12}\) particles/ml for small icosahedral viruses (Gibbs and Harrison, 1976), for a 20\(\mu l\) sample (the standard size of the wells used), the minimum amount of virus detectable by immuno-osmoelectrophoresis is \(1.3 \times 10^9\) particles. The hybridization end-point for DAV is in the region of 0.5ng of RNA (in nucleoprotein form) which is equivalent to approximately 1.8ng of virus, or \(5 \times 10^7\) particles. For DAV this represents a four hundred fold increase in the amount of virus detectable by double-diffusion in agar and a forty fold increase over immuno-osmoelectrophoresis. For DCV, hybridization increases detection sensitivity 100-200 fold above that of double-diffusion, and about a 10 fold increase over immuno-osmoelectrophoresis.
4.3.4 Comparison of Dot-Blotting Procedures

The major aim of cloning fragments of the DCV and DAV genomes was to develop a quick and sensitive assay for these viruses by utilizing cDNA hybridization. It was hoped that a detection system could be developed in which it was possible to detect the viruses in isolates from single Drosophila. To this end, various parameters were tested at three stages of the dot-blotting procedure:

(i) preparation of samples prior to blotting
(ii) the dot-blotting process
(iii) post-blotting treatment of the hybridization membrane.

4.3.4.1 Preparation of Dot-Blot Samples

Extracts of DAV and DCV were made by grinding flies known to be infected in approximately 10 times their own volume of insect saline. The serological end-point of this sample was then measured using immuno-osmoelectrophoresis. The samples were diluted to this end-point using one of the following buffers:

(i) distilled water
(ii) 15x SSC
(iii) insect saline
(iv) RNA extraction buffer (see Section 4.2.1)

Dilution series were then made using the same buffer in which they were diluted to the immunological end-point. These were then dot-blotted under vacuum as previously described (see Section 4.2.6.2C). Filters were air-dried and baked at 80°C for four hours before prehybridization and hybridization. Of the four buffers tested, distilled water and 15x SSC gave the best hybridization dilution end-points. Insect saline increased this dilution end-point by about five-fold while the RNA extraction buffer severely increased the end-point and also caused the spots to diffuse laterally. A similar increase in the hybridization
dilution end-point and lateral diffusion was seen if the dilution series were made in 0.2% w/v SDS.

As a difference was noted between the hybridization sensitivity with purified RNA and virus, a range of post-grinding treatments was tested to try and release the RNA from the virions prior to blotting. These included:

(i) Proteinase K (10mg/ml) digestion, at 56°C for 20 minutes,
(ii) incubation at 56°C for 20 minutes,
(iii) mixing samples 1:1 with chloroform and vortexing occasionally for 20 minutes,
(iv) phenol/chloroform extraction of sample (as for DNA samples, see Section 4.2.2).

None of the treatments improved the detection sensitivity for DAV or DCV above that of samples left untreated. In fact, phenol/chloroform extraction appeared to remove all of the RNA from the sample.

Therefore, the most convenient and efficient grinding system found was to grind the flies in distilled water. Diethyl pyrocarbonate (DEPC) was added to the grinding buffer to a final concentration of 0.01% to inhibit any RNases that might be present. The presence of the DEPC did not affect the hybridization sensitivity. Samples were also routinely frozen before dot-blotting. Freezing of the samples did not affect the hybridization sensitivity.

4.3.4.2 Dot-Blotting

For each single-fly isolate made it was necessary to produce two filters, one to be probed for DAV and the other to be probed for DCV. Initial experiments with single-fly isolates involved grinding each fly in 50µl of buffer and blotting 20µl onto each of two separate filters. This process was found to be unsatisfactory as, even after clarification of the sample in an Eppendorf centrifuge, large fragments of the insect
integument became stuck to the filters. These fragments would often hybridize non-specifically to the $^{32}$P-labelled probe and produce false positives.

To remedy this, samples were ground in 100µl of buffer and diluted to 200µl. 170µl of this sample was then dot-blotted onto the filters. Increasing the volume had no effect on the hybridization sensitivity but dramatically reduced the background caused by the large fragments of integument, as it was easier to avoid pipetting this material before dot-blotting.

With vacuum dot-blotting, much of the material being blotted passes through the hybridization membrane without being bound (P. Keese, pers. comm.). To test the relative efficiency of successive thicknesses of Hybond-N to trap the virus being applied, five layers of the membrane were placed in the dot-blotting apparatus. The percentage of detectable material on the five filters (from top to bottom) was distributed approximately 45, 35, 15, 5, 0. The reduction between the first and second filters is relatively small. However, as the amount of virus being applied was reduced, it appeared only to be trapped on the first and second filters, although the relative proportions on these two filters remained the same. The volume of the dot-blotted sample had no effect on the relative binding capacity of each filter for a given sample. For routine screening of single flies, it was decided to produce duplicate filters by using two thicknesses of Hybond-N in the dot-blotting apparatus. The reduction in the amount of material binding to the second filter is compensated by the increased amount of material made available by not splitting the sample into two before application to separate filters.
4.3.4.3 Post-Blotting Treatment of Hybridization Membranes

Boulton et al. (1984) reported that after squashing whole insects directly onto hybridization membranes, chloroform washing of the filter decreased the amount of background after hybridization. Chloroform washing of filters on which DAV and DCV had been vacuum dot-blotted was found to neither increase the detection sensitivity nor to decrease the background. In fact, there were indications that chloroform washing increased the background in some instances. Therefore, the hybridization membranes were not treated after blotting.

4.3.5 Detection Sensitivity in the Presence of Insect material.

Dilution series were made of unpurified virus using homogenates of virus-free *D. melanogaster*. The amount of insect material in the dilution series was calculated so that, on vacuum blotting, the equivalent of a single-fly homogenate was applied, along with the virus to each dot-blot. The immunological end-point of the unpurified virus was also measured using immuno-osmoelectrophoresis. Hybridization was found to be approximately 30-40 times more sensitive than immuno-osmoelectrophoresis in the case of DAV and slightly less for DCV. This corresponds to a detection sensitivity of approximately $4 \times 10^7$ virus particles for DAV. This detection sensitivity is in good agreement with that obtained using purified virus in the absence of insect material ($5 \times 10^7$ particles). The presence of insect material did not, therefore, reduce the sensitivity of detection.

4.3.6 A Dot-Blotting Protocol for Detecting DAV and DCV in Single *Drosophila*

The concensus protocol devised for dot-blotting of individual *Drosophila* is summarized in Figure 4.6.
Concensus dot-blotting protocol for the detection of DAV and DCV in single Drosophila

Single Drosophila

Grind in 100µl 
D. H2O, 0.01% DEPC

Dilute extract to 200µl 
with D. H2O, 0.01% DEPC

Hybond-N

Soak in 12x SSC

Load 2 thicknesses of Hybond-N 
into vacuum dot-blotter over 
2 thicknesses of blotting paper 
soaked in 12x SSC

Vacuum dot-blot 170µl of single fly extract

Air-dry Hybond-N

Bake Hybond-N at 80°C for 4 hours

Prehybridize overnight in 
40% formamide buffer (40%FB, section 4.2.6.3) 
at 65°C

Remove prehybridization buffer and add 
fresh 40%FB

Boil 32p-labelled probe 
in 5 ml of 40%FB

Add 32p-labelled probe

Hybridize overnight in 40%FB at 65°C

Wash Hybond-N twice in 2xSSC (65°C) 
and once in 0.1xSSC, 0.01% SDS (65°C)

Air-dry Hybond-N

Autoradiography
4.4 DISCUSSION

4.4.1 Protocols for Generating Specific Clones Against DAV and DCV

Two protocols were used for generating cDNA clones from DCV and DAV, and these are summarized in Figure 4.6. The reason for cloning fragments of the genomes of these two viruses was to obtain virus specific cloned cDNA fragments for a $^{32}$P-labelled DNA hybridization assay. With this aim in mind, both of the cloning protocols used in this study have advantages and disadvantages.

The original cloning method, used for both viruses, was to generate cDNA from random primed viral RNA. Double-stranded DNA was synthesised from the DNA template, BamHI linkers ligated to the dsDNA and the DNA ligated into the BamHI site of pBR322. I will refer to this as the RP method. The alternative method, used only for DCV, was to synthesize cDNA from an oligo dT (12-18) primed viral RNA. Double-stranded DNA synthesis was the same as for the RP method, but before ligation into M13, the dsDNA was restricted with a selection of restriction endonucleases with 4bp recognition sites. I will refer to this as the poly(T) method.

Using a poly(T) primer for cDNA synthesis other workers have demonstrated that it is possible to clone large fragments (2-4 K b.p.) (Cann et al., 1983) and in some cases the whole (Stanway et al., 1984) of picornavirus genomes. This priming technique has also been used to synthesise a complete copy of the RNA genome of DCV (King et al., 1984). This priming method, therefore, has the potential to generate full length cDNA from a picornavirus RNA genome. It has been noted however, that during cDNA synthesis from poly(T) primed RNA, certain subgenomic fragments of cDNA are produced in excess (King et al., 1984). This suggests that chain extension during cDNA synthesis stops preferentially at certain sites within the genome. Coupled with
the fact that chain extension also stops randomly along the RNA molecule i.e. the probability of chain extension stopping at each base-addition is approximately the same, then the overall result is that sequences towards the 5' end of the RNA molecule are progressively less well represented in the cDNA.

Random-priming on the other hand, synthesises cDNA that represents the whole of the RNA genome equally (Gould and Symons, 1977) except for the very 3' end of the genome unless an oligo-d(T) primer is used in conjunction with the random primers. One limitation with this priming method is that the length of cDNA synthesised is limited by the distance between primers. Furthermore, the method used in this study does not allow for adjacent cDNAs to be joined before progressing to a dsDNA.

The most limiting step in any ssRNA cloning procedure with respect to the size of clones generated and the relative position on the genome of those clones, is obviously cDNA synthesis. In theory, the methods used in this study should be capable of generating cDNA from: a) all regions of the genome equally (RP method), and b) regions towards the 3' end of the RNA preferentially (oligo-d(T) method).

Using the poly(T) method for cloning cDNA from DCV RNA, it was indeed found that regions towards the 3' end of the genome were preferentially cloned. The skewness of the distribution was however, greater than expected i.e. 87% of clones within 1100 bases of the virus poly(A) tail. However, as only a small amount of viral RNA was used for the cloning, and no method was utilized to optimize chain extension, sequences at the 3' end of the genome would be expected to be represented at a much higher frequency than those towards the 5' end of the molecule. The predominance of cDNA clones towards the 3' end produced using the RP method for DCV is probably due to more efficient priming of the poly(A) tail rather than internal sequences.
Nonetheless, for the purposes of this study, it was found that the RP method was able to generate relatively large, specific clones against both DAV and DCV. However, my data illustrate two possible limitations that might occur when working with small RNA viruses of insects:

(i) the viruses are in relatively short supply i.e. in this study, the amount of purified virus produced from the self-titration of 400 flies is only in the region of 60µg (equivalent to only 20µg of RNA),

(ii) virus preparations may contain more than one virus

These limitations point to the importance of choosing a cloning procedure that suits the needs of the study. Both of the cloning procedures used in this study used small amounts of viral RNA (approximately 1µg). However, by far the largest problem encountered was that of mixed virus preparations.

Although both of the virus preparations from which viral RNA were purified for cloning appeared to contain only one virus (as shown by immuno-osmoelectrophoresis), it was discovered that the DCVAN preparation did in fact contain DAV RNA. This contamination first manifested itself when it was found that cDNA made against the DCV RNA hybridized to some of the clones made from the DAV RNA. These cross-hybridizing clones were putatively assigned to a region of the DAV genome that had sufficient homology to a region in the DCV genome to cross-hybridize. However, attempted hybridizations between clones from this "cross-hybridizing region" and DCVAN RNA showed that the relative amount of hybridization did not change as the temperature increased. For this to occur, one would have to postulate that the "cross-hybridizing region" from the two genomes had virtually 100% homology. Two facts were inconsistent with this hypothesis:
(i) serologically indistinguishable strains of DCV have been found to have only 60-80% homology at the nucleotide level (King et al., 1984);

(ii) DCV and CrPV, which are serologically related, have virtually no homology at the nucleotide level (King et al., 1984) and share no large common oligonucleotides as revealed by ribonuclease T1 fingerprinting (Clewley et al., 1983).

Therefore, these two serologically unrelated viruses would have to have 100% nucleotide homology in one part of the genome i.e. at least 1200 bases (Figure 4.3), equivalent to about 15% of the total genome, while strains of DCV have as little as 60% and CrPV and DCV has less than 15% homology at the nucleotide level. Furthermore, in other regions of the genome, the nucleotide homology would have to be very low as DAV cDNA does not hybridize to any of the DCV clones and none of the DCV clones hybridize to the DAV RNA. All of these observations pointed to DAV being a contaminant of the DCVAN RNA. Confirmation of this was obtained when the two viruses were electrophoresed and transferred to membranes for hybridization (Figure 4.4C).

4.4.2 A cDNA Hybridization Assay for DAV and DCV

DAV and DCV specific cDNA clones were used to develop a cDNA hybridization detection system for both viruses. The basic detection protocol has been modified and refined so that it is capable of detecting the viruses in isolates from individual Drosophila.

Probes produced from cloned cDNAs are capable of detecting down to 0.05ng of purified viral RNA from both viruses. When the RNA is in nucleoprotein form i.e. as a virus particle, the detection sensitivity is decreased for both viruses. For DAV and DCV it is possible to detect in the region of 0.3ng and 1.2ng of RNA respectively, when they are in this form. These detection levels represent approximately 1.1ng
and 4.3ng of whole virus for DAV and DCV respectively. The difference between the detection sensitivities for the two viral RNAs when in nucleoprotein form is probably due to differences in the viruses which make the DCV RNA less available for hybridization. This could be due to a number of factors e.g. DCV binds less readily to the hybridization membrane or, once bound the viral RNA is not as readily available for hybridization as that of DAV.

The detection levels achieved for both of these viruses are comparable with those obtained in other studies using cDNA hybridization to detect structurally similar viruses. Waterhouse et al. (1986) report a detection limit of 1ng of virus particles for BYDV and Skotnicki et al. (1987) found that 0.5ng of BWYV particles could be detected using cDNA hybridization.

The dot-blotting protocol eventually adopted for the detection of DAV and DCV in isolates from single flies is shown in Figure 4.6. From the experiments outlined in this chapter, this protocol is capable of detecting approximately 3ng of DAV or 12ng of DCV in a single Drosophila.

Although problems were encountered in trying to develop an ELISA system for DAV and DCV, it was possible to detect 10-50ng/ml of CrPV using ELISA (see Section 4.1). If the same detection level could have been achieved for DAV and DCV, this would have been equivalent to detecting approximately 2-10ng of virus per individual fly. Therefore, it would appear that the cDNA detection system devised for these two viruses is at least as sensitive as ELISA.

However, the cDNA detection system has certain advantages over the ELISA system:

(i) subsequent to grinding, each sample requires very little treatment before a result is produced. With ELISA however, there are at least four reaction stages to be executed on each
sample. As it was also possible to hybridize up to 10 filters at once with no apparent reduction in the detection sensitivity, the overall saving, both in time and materials, is quite considerable using the cDNA hybridization method, although this study concentrated on DCV and DAV, the hybridization membranes are a permanent record of the viruses that are present in each fly, and so could later be re-probed for other viruses.

In conclusion then, the protocol developed for the detection of DAV and DCV in isolates from single *Drosophila* is at least as sensitive as any other *in vitro* technique and is sufficient to detect viruses in individual *Drosophila*. It is possible that the sensitivity of detection could be improved by increasing the binding of viral RNA when in the virus form e.g. pre-blotting release of the RNA from the virion. However, the protocol in its present form is simple to perform and has been shown to be suitable for screening large numbers of individual *Drosophila* in a relatively short space of time.
CHAPTER 5 STUDIES ON THE BIOLOGICAL PROPERTIES OF DAV AND DCV

5.1 INTRODUCTION

Screening of laboratory populations of *D. melanogaster* established from wild-caught flies showed that two picorna-like viruses, DAV and DCV, were common in these cultures (see Chapter 3). The initial characterisation of these two viruses was based mainly on their morphology and serological relationships, but also in part on their pathogenic effect after injection into flies using the self-titration method (Section 3.3.2).

Previous studies have shown that the picorna-like viruses of *Drosophila* can be separated into two groups on the basis of their biological properties i.e. DAV and DPV forming one group and DCV the other (see Brun and Plus, 1980 for review and Table 1.4). The laboratory populations of *D. melanogaster* established from wild-caught flies were therefore persistently infected with viruses from the two different biological groups of the *Drosophila* picorna-like viruses. Therefore, these laboratory stocks offered the opportunity to study the biological properties of the two viruses further. In particular:

(i) how are the viruses transmitted through time and space?

(ii) are there barriers to their transmission between species?

(iii) what effects do the viruses have upon the natural populations in which they occur e.g. do the viruses affect parameters such as longevity, egg production and viability?

Taken together with the data in Chapter 6 the studies concerned with these questions will help to characterise DAV and DCV at the biological level.

Two basic approaches have been used in the study of the biological properties of *Drosophila* viruses. The first is to look at the effect that the virus has after artificial transmission to virus-free flies...
e.g. either by injection or by feeding the virus to larvae or adults. As much of the biological characterisation of DAV and DCV previously reported has relied on this approach it provides information which enables the particular DAV and DCV isolates present in the laboratory stocks to be compared with other isolates of the viruses. The results of studies such as this are reported in this chapter. The second approach is to study the viruses in situ within persistently infected laboratory stocks, and to assess how they affect certain life-history and reproductive parameters. The results of these studies are reported in Chapter 6.

5.2 MATERIALS AND METHODS

5.2.1 Viruses

5.2.1.1 Source of Viruses

Reference stocks of DAV and DCV were produced by self-titration of Coffs Harbour (HD) and Ellis Beach (EB) laboratory stocks respectively (Section 3.2.2). Virus was partially purified by centrifugation through 10% sucrose (Figure 2.1, up to stage 5), and resuspended in sterile insect saline.

DCV, passed through Drosophila cells (Section 2.4.2) was passed once through the reference virus-free D. melanogaster stock (HD) and purified as described for DAV and DCV.

CrPV,BEE was obtained by passaging the virus through G. mellonella larvae. Virus was partially purified as described in Section 2.5.2, and as far as stage 5 as shown in Figure 2.1

5.2.1.2 Preparation and Storage of Virus Inocula

The serological end-point of virus preparations was measured by immuno-osmoelectrophoresis (Section 2.7.2). These preparations were
then diluted to their end-point using sterile insect saline and unwanted virus i.e. DCV contaminating DAV preparations and vice versa (see Section 3.3.3), was neutralised by adding an equal volume of the appropriate antiserum (Table 5.1)

Table 5.1 Antisera used to neutralise unwanted virus in the preparation of virus inocula

<table>
<thead>
<tr>
<th>Virus</th>
<th>Antiserum(a):Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAV</td>
<td>anti-DCV gp : 1/3</td>
</tr>
<tr>
<td>DCV</td>
<td>anti-DAV gp : 1/3</td>
</tr>
<tr>
<td>CrPV</td>
<td>anti-DAV gp : 1/3</td>
</tr>
</tbody>
</table>

(a): See Table 3.1

The virus-antiserum mixture was left for 1 hour at room temperature and the antibody-virus complexes removed by centrifugation in an Eppendorf bench-top centrifuge for 10 minutes. The supernatant was sterilized by passing through a 45 µm nitrocellulose filter. Aliquots (100µl) were stored frozen at -20°C until needed.

Neutralisation by the method described here reduced the level of unwanted virus below that detectable by dot-blot assay without affecting the titre of the virus of interest.

Virus inocula were prepared by diluting the neutralised virus preparation with freshly sterilized insect saline. The inocula were used immediately and discarded afterwards.

"Standard virus inocula" were prepared by diluting the neutralised virus preparations 1:49 with sterile insect saline. These inocula contained approximately $1.25 \times 10^9$ virus particles/ml (based on a dilution end-point of immuno-osmoelectrophoresis being equivalent to $6.25 \times 10^{10}$ virus particles/ml, see Section 4.3.3).
5.2.2 Surface Sterilization of Drosophila Eggs

Eggs were picked from the surface of artificial diet on which females had been allowed to lay overnight. The eggs were transferred to filter paper moistened with sterile insect saline and surface sterilized by dechorionation with 4% sodium hypochlorite (in sterile insect saline) for 4 minutes (Jousset and Plus, 1975). After dechorionation the eggs were washed with 100ml of sterile insect saline and transferred to fresh filter paper moistened with insect saline. The filter paper with the eggs was then transferred to fresh artificial diet.

5.2.3 Establishment of Virus-Free D. melanogaster Stocks

Virus-free stocks were established as suggested by Brun and Plus (1980). Male and female flies from a persistently infected laboratory stock were kept until all flies were at least 25 days old. Eggs were collected from these cultures and dechorionated as described in Section 5.2.2, and these eggs were allowed to hatch on fresh artificial diet. The emerging progeny were used to establish the virus-free lines. Culturing of the virus-free lines was as described in Section 2.3.2.

5.2.4 Dot-Blot Assays for DAV and DCV

Homogenates from single flies were prepared as shown in Figure 4.6. Replicate filters were made for probing by putting two thicknesses of Hybond-N in the vacuum dot-blotting apparatus (Section 4.3.4.2). Filters were treated and hybridized as shown in Figure 4.6. A standard series of controls containing $1.25 \times 10^8$, $5 \times 10^7$, $1 \times 10^7$, and $2 \times 10^6$ virus particles were spotted onto each filter.

Collected cadavers were ground in ten times their own volume of distilled water, 0.01% DEPC. The homogenate was clarified by
In all experiments reported in this chapter an experimental "group" comprised 100 injected flies.
centrifugation and 20µl of the supernatant dot-blotted as described for homogenates from single flies. Filters were treated and hybridized as shown in Figure 4.6.

5.2.5 Measurement of Mortality Parameters from Injection Experiments

In all experiments on the effects of injecting virus into adult Drosophila, a control sample of flies was always injected with insect saline at the same time that flies were injected with virus. Injected flies were transferred every 3-4 days to fresh artificial diet and dead flies were counted and collected. Experiments were generally terminated after 27-30 days, or when the mortality was estimated to be in excess of 90%. Cumulative percentage mortality was calculated for each group of flies. Flies that died after injection of saline in the period 0-3 days after injection were deemed to have died from either shock or microbial contamination caused by the needle piercing the integument. These flies were removed from the analysis and the numbers in the virus injected groups were adjusted by this amount. The difference in cumulative percentage mortality between the virus-injected and saline-injected flies (Δ C.P.M., see Figure 5.1A) was calculated and used to estimate the parameter T_{50} - the time at which 50% of the population were dead (Figure 5.1B).

As shown in Figure 5.1B, Δ C.P.M. values tend to reach a maximum and then decrease. The Δ C.P.M. decrease occurs when the increase in control group (saline injected) mortality is greater than the increase in virus-injected group mortality. In experiments where T_{50} was estimated from a linear regression of Δ C.P.M. on time (or transformed time data e.g. see Section 5.3.3), regressions were calculated only to the time when Δ C.P.M. attained its maximum value. Any virally-induced mortality effects occurring after this time are obscured by the endogenous mortality in the control group.
The figure presented opposite (Figure 5.1) is derived from consensus data and is designed to show the methods whereby ΔCPM, $T_{50}$ and $M_{24}$ were derived for analyses in both Chapters 5 and 6. The graphical method used for the derivation of the latter two values was applied only to experiments reported in Chapter 6. All $T_{50}$ values presented in the current chapter were calculated as described on p95 (see also Section 5.3.3).
Figure 5.1 Estimation of the parameters $\Delta$ C.P.M. (A) and $T_{50}$ and $M_{24}$ (B)

The experiments described in this section involve the transplacental transfer of the virus SA IV and SA IV by artificial insemination. In all of these experiments, a stock of flies was used derived from the Coffin strain and females of greater than 75 days of age were used. In the experiments 1, 2, 3 and 4, groups of flies were injected with approximately 2000 viral particles. The results presented in Figures 5.1A and 5.1B demonstrate how the virus is retained by the host after infection with SA IV. The specific activity of the virus decreases with time after infection.
5.3 RESULTS

The experiments described in this section involve the transmission of the viruses DAV and DCV by artificial means to various species of Drosophila i.e. injection into adults, or *per os* to larvae. In all of these experiments in which *D. melanogaster* is involved a reference virus-free stock of flies were used - HD<sub>ss</sub>. This stock was derived from the Coffs Harbour (HD) stock by surface sterilization of eggs from old females of greater than 20 days of age (see Section 5.2.2 and 5.2.3).

5.3.1 Injection of DAV and DCV into *D. melanogaster* and *D. simulans*

Groups of flies from a *D. simulans* stock established from flies collected in Canberra that did not produce any detectable virus after self-titration, and the virus-free reference stock of *D. melanogaster* (HD<sub>ss</sub>), were injected with standard inocula of DAV and DCV. Control groups of flies from these two stocks were injected with sterile insect saline. Mortality in each group of injected flies was monitored every 3-4 days, and the cadavers were collected and stored at -20°C until they were screened for DAV or DCV. Surviving flies were killed and stored at -20°C. Cumulative percentage mortality data was corrected as described in Section 5.2.5.

The corrected time mortality response curves for DAV injected flies are presented in Figure 5.2A. These data demonstrate two features of the response of *D. melanogaster* and *D. simulans* to injection with DAV. Firstly, there is no significant difference between *D. melanogaster* and *D. simulans* in their response to injection with DAV. Second, the shape of the response curves is sigmoidal/parabolic. This shape suggests that the mortality response of *D. melanogaster/simulans* to DAV is limited to the first 15-20 days post-injection i.e. after this time the curve begins to plateau. DAV
Figure 5.2 Cumulative percentage mortality curves for *D. melanogaster* (x—x) and *D. simulans* (o—o) injected with DAV (A) and DCVEB (B).

A. C.P.M. (%)

B. C.P.M. (%)

Experimental mortality response curves of DCV injected *D. melanogaster* and *D. simulans* are presented in Figure 5.2. The mortality response of both species to DCV is similar, with a delay of at least 4 days before a high level of mortality is reached. These results are in agreement with previous data (15) and Neun, 1940, which suggests that the high mortality in DCV-infected flies is not due to the virus itself but to an interaction between the virus and the host.
was detectable in the cadavers of all flies collected from 6 days onwards. Furthermore, all flies surviving to 30 days were found to contain high titres of the virus. Therefore, it would appear that DAV is able to replicate quite freely in *D. melanogaster* and in *D. simulans* after injection but has relatively low pathogenicity.

Experiments were carried out to ascertain how long after injection DAV reached levels that were detectable by the dot-blot assay. Samples from a group of injected flies were removed at 2, 5, 8, and 11 days and screened individually. After 2 days the infection frequency was found to be 67%. At day 5, and later, all of the flies tested were found to be infected. As the first mortality effects due to injection of the virus were not detectable until 6 days this suggests that there is a delay of at least 4 days between the virus reaching detectable levels and flies dying from its effects.

The corrected time-mortality response curves of DCV injected *D. melanogaster* and *D. simulans* are presented in Figure 5.2B. The mortality response of both species to DCV is much greater than it is to DAV. Maximal mortality for both species is reached at about 10 days. These results are in agreement with previous data (e.g. see Plus and Brun, 1980) which showed that DCV has higher pathogenicity than DAV both for *D. melanogaster* and *D. simulans*.

### 5.3.2 Titration of DAV and DCV in *D. melanogaster*

Virus preparations were prepared as described in Section 5.2.1.2. Inocula containing $3 \times 10^{10}$ particles/ml were diluted 1:9, 1:19, 1:49 and 1:199 with sterile insect saline. Each of these dilutions were injected into a separate group of virus-free *D. melanogaster* (reference stock, HD$_{ss}$) and the mortality monitored as described in Section 5.2.5.
5.3.2.1 Titration of DAV

Corrected mortality-time response curves were plotted for each group of flies. Maximal mortality occurred for each dilution of inocula at approximately 16 days. However, the maximal mortality level was not the same for each dilution of virus tested. A similar pattern of response was seen when the titration was carried out in virus-free flies from the Ellis Beach stock, EB$_{50}$ (data not presented). Corrected cumulative percentage mortality ($\Delta$ C.P.M.) was plotted against log transformed dilution values, Figure 5.3A. The relationship between $\Delta$ C.P.M. and log virus dilution is not linear. Maximum mortality occurs with a 1 in 50 dilution of the virus preparation ($6 \times 10^8$ particles/ml), and mortality decreases as the virus concentration is increased. This suggests that at virus concentrations greater than those which produce the largest pathogenic effect some factor is inhibiting the pathogenic effect of the virus.

5.3.2.2 Titration of DCV

Corrected mortality time response curves were plotted for each group of injected flies. T$_{50}$ and $\Delta$ C.P.M. were plotted against log transformed inocula dilutions (Figure 5.3B and C). Contrary to the titrations of DAV, these data show that there is a positive linear relationship between mortality and virus concentration.

5.3.3 Comparison of the Pathogenicity of DCV$_{EB}$, DCV$_{O}$ and CrPV$_{BEE}$

Previous data on the pathogenicity of DCV show that the virus, when injected, causes 100% mortality in less than 4 days (Jousset et al., 1972). Under the conditions used in this study, such high mortality was not produced by DCV$_{EB}$. This relative decrease in pathogenicity could be due to two factors. Firstly, it is possible that DCV$_{EB}$ is less pathogenic after injection than most overseas
Figure 5.3 Titration of DAV and DCV in D. melanogaster. Mortality parameters (Δ C.P.M. and T₅₀) are plotted against log of virus inocula dilution (v.i.d.)

A. Titration of DAV in D. melanogaster. Δ C.P.M. is plotted against v.i.d. at various times after injection:

- x—x = 9 days
- o—o = 12 days
- *—* = 15 days
- ^—^ = 18 days

B. Titration of DCV in D. melanogaster. T₅₀ is plotted against v.i.d.

C. Titration of DCV in D. melanogaster. Δ C.P.M. is plotted against v.i.d. at various times after injection:

- x—x = 9 days
- o—o = 12 days
- *—* = 15 days
Figure 5.3 Titration of DAV and DCV in *D. melanogaster*

A. 

Δ C.P.M. (%) 

- 30
- 20
- 10
- 0

log(v.i.d.)

B. 

T50 (Days)

- 10

- 1

log(v.i.d.)

C. 

Δ C.P.M. (%) 

- 80
- 60
- 40
- 0

log(v.i.d.)
isolates of DCV. Second, the observed lower pathogenicity may be due to increased resistance of the HD$_{ss}$ reference stock to injection with DCV relative to the reference Champetieres stock used in previous studies. To test these alternatives the virus-free reference stock (HD$_{ss}$), and a virus-free stock produced from the Ellis Beach stock (EB$_{ss}$) were each injected with standard isolates of DCV$_{EB}$ and DCV$_0$. Each isolate was estimated by immuno-osmoelectrophoresis (see Section 2.7.2) to contain 1.25x10$^9$ virus-particles/ml. As a further control, flies from both virus-free stocks were also injected with an isolate of CrPV$_{BEE}$ that contained 1.25x10$^9$ virus particles/ml. Mortality was monitored as described in Section 5.2.5. A C.P.M. was calculated and plotted against log transformed-time data. A simple regression was fitted to these data and the parameters $T_{50}$ and b (slope of the regression) were calculated (Table 5.2).

Table 5.2 $T_{50}$ and b for HD$_{ss}$ and EB$_{ss}$ injected with DCV$_{EB}$, DCV$_0$ and CrPV$_{BEE}$. Each $T_{50}$ value presented is for a single group of 100 flies.

<table>
<thead>
<tr>
<th>Stock</th>
<th>DCV$_{EB}$</th>
<th>DCV$_0$</th>
<th>CrPV$_{BEE}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD$_{ss}$</td>
<td>$T_{50}$</td>
<td>9.62</td>
<td>9.48</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>104.92</td>
<td>98.75</td>
</tr>
<tr>
<td>EB$_{ss}$</td>
<td>$T_{50}$</td>
<td>9.12</td>
<td>8.86</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>99.94</td>
<td>92.59</td>
</tr>
</tbody>
</table>

These data show that there is very little difference in the pathogenicity of DCV$_{EB}$ and DCV$_0$. The $T_{50}$ values obtained for both viruses are, however, much higher than previously reported i.e. DCV$_0$ usually produces 100% mortality in under 4 days ($T_{50}<3$ days). A high mortality usually results even if the titre of the injected virus is
much lower (i.e. $10^8$ virus particles/ml) than the $1.25 \times 10^9$ particles/ml used here (Plus et al., 1978). However, it should be noted that the above experiments were done at 21-22°C rather than 25°C (Plus et al., 1978), which may account for the decreased pathogenicity of DCV when compared with previous reports. Notwithstanding the lower temperature used in these experiments, it is notable that the pathogenicity of CrPV is greater than that of either of the DCV isolates. This is contrary to previous studies, in which it was shown that the pathogenicity of CrPV was lower than that of DCV (Plus et al., 1978). Therefore, if incubation temperature affects the pathogenicity of DCV it does not affect CrPV to the same extent.

5.3.4 Geographical Variation in Susceptibility of D. melanogaster Stocks to Injection with DCV.

The data (Table 5.2) show that there is a slight difference in the pathogenicity of DCV to the virus-free stocks established from Ellis Beach (EB$_{ss}$) and from Coffs Harbour (HD$_{ss}$) laboratory populations. To investigate this difference further, experiments were performed in which flies from both of these virus-free stocks, together with virus-free flies from the Huonville (HV) laboratory population, were injected with the same standard isolate of DCV$_{EB}$. The data were transformed as described in section 5.3.3 and $T_{50}$ and $b$ were calculated (Table 5.3).

The $T_{50}$ obtained from the EB$_{ss}$ injected flies was consistently lower than that obtained from the HD$_{ss}$ flies. However, the mean $T_{50}$ in the two stocks was not significantly different ($t_{[6]}=1.15$, $p>0.5$) (Table 5.3).
Table 5.3 $T_{50}$ and simple regression coefficients ($b$) from regressions of Δ C.P.M. on log transformed time for DCV injected HD$_{ss}$ and EB$_{ss}$ virus-free stocks

<table>
<thead>
<tr>
<th>Experiment</th>
<th>$T_{50}$</th>
<th>$b$</th>
<th>$T_{50}$</th>
<th>$b$</th>
<th>$T_{50}$</th>
<th>$b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>16.00</td>
<td>125.50</td>
<td>16.00</td>
<td>9.66</td>
<td>110.42</td>
<td>115.33</td>
</tr>
<tr>
<td>2.</td>
<td>12.28</td>
<td>100.61</td>
<td>9.66</td>
<td>8.14</td>
<td>142.58</td>
<td>99.94</td>
</tr>
<tr>
<td>3.</td>
<td>116.00</td>
<td>110.42</td>
<td>116.00</td>
<td>9.12</td>
<td>142.58</td>
<td>99.94</td>
</tr>
<tr>
<td>4.</td>
<td>122.63</td>
<td>115.33</td>
<td>122.63</td>
<td>99.94</td>
<td>144.92</td>
<td>(a)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>HD$_{ss}$</th>
<th>EB$_{ss}$</th>
<th>HV$_{ss}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>11.01</td>
<td>8.50</td>
<td>-</td>
</tr>
<tr>
<td>SD</td>
<td>3.34</td>
<td>2.85</td>
<td>-</td>
</tr>
<tr>
<td>Mean</td>
<td>118.47</td>
<td>122.01</td>
<td>-</td>
</tr>
<tr>
<td>SD</td>
<td>6.85</td>
<td>25.12</td>
<td>-</td>
</tr>
</tbody>
</table>

Comparison of mean $T_{50}$ between HD$_{ss}$ and EB$_{ss}$ stock: 
$F_{[3,3]} = 1.37$, $p>0.5$

Comparison of mean $b$ between HD$_{ss}$ and EB$_{ss}$ stocks: $F_{[6]} = 13.38$, 0.1 $p>0.05$

(a) Only one replication of the experiment was possible using HV$_{ss}$ flies as the stock had become infected with DAV by experiment 2 (see Section 6.3.2.1D)
5.3.5 Replication of DAV and DCV in Species Other than *D. melanogaster* and *D. simulans*

Groups of *D. hydei*, *D. immigrans*, *D. sulfurigaster* and *D. ananassae* were injected with standard inocula of DAV and DCV. Control groups of flies of each species were injected with sterile insect saline. Mortality in each group of injected flies was monitored as described in Section 5.2.5.

The time-mortality response curves for each species are presented in Figure 5.4. No differences between the virus injected groups of flies and the saline injected controls are apparent for either *D. ananassae* or *D. sulfurigaster*. Note, however, that for these two species the mortality of the saline controls is very high after 3 days, and this might obscure any pathogenic response to the virus. In *D. hydei* and *D. immigrans* there are slight differences in mortality between the groups of flies injected with saline and those injected with DAV and DCV. However, the pathogenic response due to injection of DCV in these species is very much lower than that observed in *D. melanogaster* and *D. simulans*.

To test whether DAV could replicate in these species, cadavers collected during the course of the experiment, and individuals surviving at the termination of the experiment, were screened for the virus. The results of this screening are summarized in Table 5.4.

Although DAV does not cause a strong pathogenic effect it was detected, and thus is able to replicate, in *D. hydei*, *D. immigrans*, *D. ananassae* and *D. sulfurigaster*. A slight pathogenic effect was found in *D. hydei* and *D. immigrans* after injection of DAV. However, there was no evidence that DCV replicated in any of the species tested as DCV was not detected in the cadavers collected from any of the groups of flies injected with DCV or from any of the flies surviving at the termination of the experiments.
Figure 5.4 Cumulative percentage mortality curves for *D. hydei*,
*D. immigrans*, *D. sulfurigaster* and *D. ananassae* injected
with saline, DCV_{EB} and DAV

- Saline injected....x--x
- DCV_{EB} injected.....o--o
- DAV injected.......*--*
Figure 5.4 Cumulative percentage mortality curves for *D. hydei*, *D. immigrans*, *D. sulfurigaster* and *D. ananassae* injected with saline, DCVEB and DAV.
Figure 5.4 ......continued.

C.P.M. (%)

0 10 20 30 Time (Days)

D. sulfurigaster

C.P.M. (%)

0 10 20 30 Time (Days)

D. ananassae

[Graphs showing the percentage of C.P.M. for D. sulfurigaster and D. ananassae over time.]
Table 5.4 Presence of DAV in collected cadavers and survivors of species other than *D. melanogaster* and *D. simulans*

<table>
<thead>
<tr>
<th>Species</th>
<th>DAY&lt;sup&gt;a&lt;/sup&gt;</th>
<th>3</th>
<th>7</th>
<th>10</th>
<th>14</th>
<th>17</th>
<th>20</th>
<th>24</th>
<th>SURV&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. hydei</em></td>
<td></td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>D. immigrans</em></td>
<td></td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><em>D. sulfurigaster</em></td>
<td></td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td><em>D. ananassae</em></td>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> days after injection; <sup>b</sup> surviving flies at termination of experiment; + = virus detected; - = virus not detected; 0 = experiment terminated prior to this day.

5.3.6 Effect of DAV and DCV on Larval Survival

5.3.6.1 Artificial Contamination of Diet

To test for any effect of DAV and DCV on larval viability, first instar larvae were reared on contaminated diet. A known number of eggs (between 50-60) from the reference virus-free stock (*HD<sub>ss</sub>*<sup>s</sup>) were transferred to fresh *Drosophila* diet on sterile filter paper. The eggs were allowed to hatch overnight, the filter paper removed, and unhatched eggs and larvae retained on the filter paper were counted and subtracted from the number of eggs originally transferred. The diet was then contaminated with 1ml of a virus inoculum containing 3x10<sup>9</sup> virus particles/ml, or treated with sterile insect saline. Larvae were allowed to develop and the progeny collected every day for ten days after the first adults had emerged. At least 5 replicates were set up for each treatment. The proportion of flies surviving through to the imago stage for each treatment is shown in Table 5.5.

An analysis of variance of these data showed that the mean proportions did not differ significantly between treatments \(F_{(2,13)} = 1.83, 0.5>p>0.2\), and thus it appears that contamination of larval diet with DAV and DCV has no effect on the viability of larvae.
Table 5.5 Viability of larvae reared on DAV or DCV infected diet

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DAV</th>
<th>DCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.897</td>
<td>0.912</td>
<td>0.814</td>
</tr>
<tr>
<td>SD</td>
<td>0.042</td>
<td>0.051</td>
<td>0.109</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

5.3.6.2 Viability of Larvae from Persistently Infected Mothers.

Two groups of approximately 50 eggs were picked from the surface of diet on which 6-7 day old females had been allowed to lay their eggs. One group of eggs was washed in insect saline, counted and transferred to a bottle of fresh diet. The other group of eggs was dechorionated as described in Section 5.2.2, counted and transferred to a bottle of fresh diet. The diet on which the eggs were placed was seeded with a small amount of dried bakers yeast. Progeny were collected as described in Section 5.3.6.1.

Comparisons for each treatment were made between a laboratory stock persistently infected with DAV (HD) and a virus-free line derived from the same stock (HD₃₃), and between a laboratory stock persistently infected with DCV (EB) and a virus-free line derived from that stock (EB₃₃). The results of these tests are summarized in Table 5.6. No assessment was made of the fertility or hatchability of the eggs in this experiment as in most cases the unhatched eggs on the filter paper became covered in a layer of yeast. Therefore, the results summarized in Table 5.6 represent the cumulative proportions of flies surviving from egg to imago.

Between the stock persistently infected with DAV and its corresponding control, significant differences were found in the proportion of imagos produced by the two treatments (Table 5.6) (HD - F[7,7]=1.847, t[14]=2.312, 0.05>p>0.02; HD₃₃ - F[3,3]=2.72, t[6]=2.540, 0.05>p>0.02). However, no difference was found between the
Table 5.6 Viability of eggs produced by *D. melanogaster* persistently infected with DAV or DCV

A. DAV Infected Mothers; Coffs Harbour (HD) stock and control virus-free stock (HD<sub>ss</sub>)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Infected Mothers</th>
<th>Control Mothers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Hypochlorite</td>
</tr>
<tr>
<td>Mean</td>
<td>0.879</td>
<td>0.786</td>
</tr>
<tr>
<td>SD</td>
<td>0.064</td>
<td>0.088</td>
</tr>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

B. DCV Infected Mothers, Ellis Beach stock (EB) and control, virus-free stock (EB<sub>ss</sub>)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Infected Mothers</th>
<th>Control Mothers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Hypochlorite</td>
</tr>
<tr>
<td>Mean</td>
<td>0.881</td>
<td>0.811</td>
</tr>
<tr>
<td>SD</td>
<td>0.035</td>
<td>0.063</td>
</tr>
<tr>
<td>n</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>
two treatments for the persistently DCV-infected stock and its corresponding control (EB - $F_{[3,3]}=3.320$, $t_{[6]}=1.918$, $0.2>p>0.05$; $EB_{ss} - F_{[3,3]}=6.083$, $t_{[6]}=2.228$, $0.2>p>0.05$).

Comparisons for each treatment between the virus-infected lines and their corresponding controls revealed no significant differences:

- DAV lines saline treated: $F_{[7,3]}=0.998$, $t_{[10]}=0.897$, $0.5>p>0.2$
- DAV lines hypochlorite treated: $F_{[7,3]}=1.290$, $t_{[10]}=0.502$, $p>0.5$
- DCV lines saline treated: $F_{[3,3]}=1.533$, $t_{[6]}=0.108$, $p>0.5$
- DCV lines hypochlorite treated: $F_{[3,3]}=2.810$, $t_{[6]}=0.871$, $0.5>p>0.2$

The decreased proportion of eggs producing imagos after hypochlorite treatment (in all stocks) is undoubtedly due to the severity of the dechorionation process. However, the homogeneity of the proportions of eggs producing imagos between the virus infected and control stocks (regardless of treatment) shows that the presence of DAV or DCV within these stocks does not affect egg to adult viability.

5.3.7 Contact Transmission of DAV Between Adult D. melanogaster

Flies from the virus-free reference stock (HD$_{ss}$) and the persistently DAV-infected laboratory stock (HD) were lightly etherised and sorted into males and females. Replicates of 25 males from the virus-free stock and 25 females from the persistently infected stock were transferred to fresh artificial diet. Similar sets of the reciprocal types (25 HD$_{ss}$ females and 25 HD males) were also set up. One replicate from each of the reciprocal groups was harvested at 3, 9, 12, 15 and 21 days after the start of the experiment. Individual flies were screened for DAV as described in Section 5.2.4. Control groups of 50 HD$_{ss}$ flies and 50 HD flies were kept concurrently with the above groups of flies and harvested at 21 days. Individual flies were screened for DAV.
Figure 5.5 Contact transmission of DAV between infected and uninfected D. melanogaster

A. Transmission between females from the persistently infected stock (HD) (x-x) to males from the uninfected stock (HD_{ss}) (o--o). I.F. = infection frequency.

B. Transmission between males from the persistently infected stock (HD) (o--o) to females from the uninfected stock (HD_{ss}) (x-x). I.F. = infection frequency.
Figure 5.5 Contact transmission of DAV between infected and uninfected
D. melanogaster

A.

I.F. (%)  

<table>
<thead>
<tr>
<th>Time (Days)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>20</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
<td>40</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>20</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>20</td>
<td>40</td>
<td>60</td>
</tr>
</tbody>
</table>

B.

I.F. (%)  

<table>
<thead>
<tr>
<th>Time (Days)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>20</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
<td>40</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>20</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>20</td>
<td>40</td>
<td>60</td>
</tr>
</tbody>
</table>

The frequency of infected males and females in each group of flies exposed to virus in Figure 5.5. DAV was not detected in the flies of the control group, while the infection frequency in males and females in the infected group was found to be 20% at 21 days. From Figure 5.5 we can deduce the following about the contact transmission of DAV between males and females:

(i) Males transmit the virus to females and females transmit the virus to males.

(ii) Males acquire a detectable level of virus earlier than 12 days whereas females (40 days). Thus, in the presence of the opposite sex, the time of acquisition of the virus is the time that it takes for the virus to be transmitted and then to replicate to a detectable level. Therefore, we could say that the infection frequency in the persistently infected culture was transmitted by contact soon after they emerge. However, the experiments were established from flies that were 3-4 days old and therefore were flies which had only been exposed to the virus or infected stock flies which were concluded to represent those flies collected in the group which were 5-6 days old. So, it would make sense that not every fly that emerges from a persistently infected stock is infected.
The frequency of infected males and females in each group of flies is summarized in Figure 5.5. DAV was not detected in the HD\textsubscript{s}s controls, while the infection frequency both in males and females in the HD control group was found to be 98% at 21 days.

From Figure 5.5 we can deduce two features about the contact transmission of DAV between males and females:

(i) males transmit the virus to females and females transmit the virus to males,

(ii) males acquire a detectable level of virus after a shorter time (12 days) than do females (15 days), when in the presence of the opposite sex.

The acquisition time for the virus is the time that it takes for the virus to be transmitted and then to replicate to a detectable level. Therefore, it would seem that the infection frequency in the persistently infected sex immediately prior to the time at which the virus is first detected in the virus-free flies is a reflection of the proportion of flies in the persistently infected stock that are infected when the experiment is started i.e. those that are infected at eclosion. The possibility exists that flies that emerge uninfected from the persistently infected culture become infected by contact soon after they emerge. However, the experiments were established from flies that were 0-1 days old and therefore these flies would only be detectable as being infected 1 day prior to those that originally came from the uninfected stock. Hence, they would not be included in the group which were concluded to represent those flies infected at eclosion. So, it would appear that not every fly that emerges from a persistently infected stock is infected.
5.4 DISCUSSION

DAV proved capable of replicating readily in both *D. melanogaster* and *D. simulans*. As has previously been reported (see Brun and Plus, 1980), DAV has a relatively low pathogenicity after injection. Very little difference could be detected in the relative susceptibility of *D. melanogaster* and *D. simulans* to injection with DAV, and maximal mortality for both species was limited to 20-30%. The shape of the mortality-response curves (see Figure 5.2) suggested that the pathogenicity of DAV for *D. melanogaster* and *D. simulans* was relatively slow-acting, and the effects of the virus were not noticeable prior to 6-9 days post-injection. However, growth of the virus was relatively rapid and only 2 days after receiving approximately $6 \times 10^5$ particles of virus ($0.5\mu l$ of virus inoculum containing $1.25 \times 10^9$ particles/ml), 70% of the injected flies were found to contain at least $1 \times 10^{10}$ particles. Even though high levels of virus were detectable such a short time after injection, the mortality of the treated flies did not rise above that of the saline injected controls until at least 6 days post injection. Furthermore, virus was not detected in collected cadavers until 6 days after injection. Presumably virus was not detected in cadavers prior to 6 days because the flies that died by that time had died from the effects of the injection itself, and not from the effects of the virus per se. Therefore, even after the virus reaches a high titre in injected flies there is a delay of at least 4 days before any mortality effect due to the injection of the virus can be detected.

Titration of DAV in *D. melanogaster* was found not to produce a linear relationship between log inocula concentration and mortality in *D. melanogaster* (see Figure 5.3). This result suggests that the virus is self-inhibitory when injected in concentrations above $1.25 \times 10^9$ particles/ml. However, it is also possible that the antisera present
in the inocula (see Section 5.2.1.2) are playing some non-specific inhibitory role, even though no effect was detected in controls injected with a high concentration of anti-DCV gp serum. If decreased mortality at high inoculum concentrations is real, then this is the first report of such an effect for small RNA viruses of insects. Regardless of the causative mechanism of the decrease in mortality, this topic obviously warrants more research.

The host range of DAV has not previously been investigated. Results presented in this chapter (see Section 5.3.2.6) demonstrate that DAV is capable of replicating in all species tested -

D. melanogaster, D. simulans, D. hydei, D. immigrans, D. sulfurigaster and D. ananassae. These species come from two of the major subgenera of Drosophila, Sophophora (D. melanogaster, D. simulans and D. ananassae) and Drosophila (D. immigrans, D. sulfurigaster and D. hydei). A slight pathogenic effect following injection of DAV is detectable in D. hydei and D. immigrans. If a similar effect exists in D. sulfurigaster and D. ananassae then it was obscured by the high mortality of saline injected controls (see Figure 5.4).

Jousset and Plus (1975) demonstrated that DPV was transmissable to larvae by contamination of the diet, and that the virus caused a significant decrease in the viability of larvae infected in this way. DPV and DAV have, as far as is known, identical biological properties (see Table 1.4) and Jousset and Plus considered them to be different serotypes of the same virus. In my experiment DAV did not decrease the viability of larvae reared on diet infected with the virus. In addition, eggs from females that were persistently infected with DAV did not show any reduced viability compared with eggs from virus-free mothers of the same stock. So, under the conditions employed in the present study DAV does not appear to have any direct effect on larval viability.
DCV has previously been shown to be highly pathogenic after injection into *D. melanogaster* (Jousset et al., 1972). Injection of an isolate prepared from the persistently DCV-infected Ellis Beach stock, DCV<sub>EB</sub>, was found to be more pathogenic than DAV upon injection into *D. melanogaster* and *D. simulans* (Figure 5.2). However, the pathogenicity of this isolate of DCV appeared to be less than that previously reported for a range of DCV isolates (Plus et al., 1978). A comparison of the DCV<sub>EB</sub> and the highly pathogenic DCV<sub>0</sub> revealed no apparent differences after injection into either the Coffs Harbour or Ellis Beach virus-free stocks (HD<sub>ss</sub> and EB<sub>ss</sub>). The observed pathogenicity of DCV<sub>0</sub> was, however, lower than previously reported for this particular isolate. This result suggests that the HD<sub>ss</sub> and EB<sub>ss</sub> stocks are more resistant to DCV than the Champetieres stocks used in previous studies (e.g. Plus et al., 1978), although experiments in the current study were carried out at 21-22°C rather than 25°C. Notwithstanding this difference in experimental temperature, the T<sub>50</sub> produced by injecting HD<sub>ss</sub> and EB<sub>ss</sub> with CrPV<sub>BEE</sub> was in the region of 6-7 days. This value of T<sub>50</sub> is in good agreement with that previously reported for CrPV ((Plus et al., 1978; Plus and Scotti, 1984). The response of the local virus-free strains of *D. melanogaster* to injection with CrPV therefore appears to be the same as previously studied strains of *D. melanogaster* (i.e. Champetieres), while they differ in their response to injection with DCV. Therefore, whatever factor(s) are responsible for the observed reduction in pathogenicity of DCV isolates after injection into local virus-free strains of *D. melanogaster*, they do not affect CrPV in a similar fashion. The pathogenic response of local virus-free strains of *D. melanogaster* after injection with DCV isolates was also variable (see Table 5.3). From the available data, it would appear that the reduced pathogenicity of DCV after injection into local virus-free strains of
D. melanogaster is due the presence of resistance factors. These putative resistance factors would not, however, confer resistance against CrPV despite the apparent close relationship between DCV and CrPV in terms of their biological properties (see Scotti et al., 1981).

The nature of these putative resistance factors is of interest because such factors that have previously been described in D. melanogaster are linked to l(2)gl lethals and act through cytoplasmic factors. In contrast, the resistance factors identified against sigma virus are semi-dominant genes located on each of the 3 major chromosomes of D. melanogaster (Gay, 1978). One of the resistance factors against sigma virus, ref(2)P, is polymorphic and has been found to be geographically correlated with a polymorphism in sigma virus (Fleuriet, 1986).

Jousset (1976) demonstrated that the pathogenic effect of DCV, after injection, decreased with taxonomic distance from D. melanogaster and D. simulans. A slight pathogenic response was detectable in D. immigrans and the virus was shown to replicate to approximately 10^7 particles/fly. The present study was able to identify a slight pathogenic response to DCV, after injection, in D. immigrans and D. hydei but not in D. ananassae or D. sulfurigaster. Although DCV was readily detectable in cadavers collected from groups of D. melanogaster/simulans injected with DCV, the virus could not be detected in cadavers collected from any of the other species that were injected. However, it should be remembered that the detection level for DCV in the dot-blot assay used in the present study is in the region of 2x10^8 particles. From the results of Jousset (1976), it would be unlikely that DCV would be detected in species other than D. melanogaster and D. simulans by using the dot-blot assay developed in the present study, as the virus does not replicate to this level in D. immigrans.
Although DCV was found to have relatively high pathogenicity after injection into adult *D. melanogaster*, per os infection of larvae did not significantly reduce their viability. This result is contrary to the report of Jousset and Plus (1975), who found that larvae reared on diet contaminated with DCV had reduced viability. In addition, no significant reduction was found in the viability of eggs produced by DCV infected mothers. So, under the conditions used in the current study, and using local virus-free strains of *D. melanogaster*, no reduction of larval viability could be identified as being associated with DCV. Again, the presence of resistance factors that function during the larval stages might be implicated.
CHAPTER 6 STUDIES ON LABORATORY STOCKS OF D. MELANOGASTER

PERSISTENTLY INFECTED WITH DAV AND DCV

6.1 INTRODUCTION

Few studies have concentrated on the effects that the picorna-like viruses of Drosophila have upon their hosts. The laboratory stocks established from natural populations of D. melanogaster during the course of the present study offered an opportunity to study the effects that DAV and DCV have upon D. melanogaster. Three areas were considered for particular investigation:

(i) adult survival
(ii) egg to adult viability
(iii) egg production and fertility

These three areas were chosen as previous studies have indicated that each may be affected by small RNA virus infections. David and Plus (1971) showed that DPV was associated with slight reductions in longevity (of males and females) and egg production in D. melanogaster. RNA viruses of insects have been found to be associated with reductions in the life expectancy and egg production of the host (e.g. D'Arcy et al., 1981b) and can have severe effects on the development and pre-adult survival of the host (Chao et al., 1983).

6.2 MATERIALS AND METHODS

6.2.1 Measurement of Life-History, Reproductive and Physical Parameters

6.2.1.1 Mortality

In the mortality experiments flies were collected and aged as described in Section 3.2.2.1. All experiments were initiated with
flies that were between 0 and 2 days old. Flies were transferred to fresh artificial diet every 3-4 days and the dead counted and stored at -20°C. Experiments were generally terminated between 27-30 days and the surviving flies harvested and stored at -20°C. Cumulative percentage mortality was calculated and two parameters estimated graphically: $T_{50}$ and $M_{24}$ - mortality at 24 days, Figure 5.1B.

6.2.1.2 Egg Production

Flies were maintained in 250ml bottles, and mortality measured as described in 6.2.1.1, except that male and female mortality were scored separately. However, after 3 days on one bottle of diet flies were aspirated into three 60ml specimen containers containing freshly prepared artificial diet. The numbers of flies in each of the specimen tubes were approximately equal. The flies were left on the diet for 24 hours and then transferred back into a 250ml culture bottle. The eggs in the specimen tubes were counted, and the total number of eggs for each group (3 specimen tubes) was calculated. Measurements were repeated as described above i.e. one measurement every 4 days, until the experiment was terminated.

6.2.1.3 Winglength

Winglength was used as an index of adult body size in D. melanogaster as it is the easiest morphometric trait to measure on large numbers of individuals. The left wing of dead D. melanogaster were removed and stuck to double sided transfer tape. The winglength was measured along the third longitudinal vein from the wing-tip to the anterior crossvein (Atkinson, 1979b). Female winglength was generally used as an index because a strong linear positive relationship was found between male and female wing length (Simple correlation coefficient $r_{[18]} = 0.927, p<0.001$).
Note: all measurements of winglength given in this study are in graticule units. Five graticule units = 1 mm, n (for each graph) ≥ 24

6.2.2 Statistical Tests

All statistical tests were calculated as described in Zar (1984) and Sokal and Rohlf (1981).

6.3 RESULTS

6.3.1 Characterisation of the Viruses Present in Persistently Infected Laboratory Stocks of D. melanogaster

Six laboratory stocks of D. melanogaster established from wild-caught flies were used in the work described in this chapter; Ellis Beach, north Queensland (EB); Cardwell (Mangoes), north Queensland (CaM); Coffs Harbour, northern N.S.W. (HD); Araluen, southern N.S.W. (AN); Tamar Valley, Tasmania (TR) and Huonville, Tasmania (HV) (see Table 7.1 for further details of the original collection sites).

The experiments reported in this chapter were done in two periods; August-November, 1985 and August-December, 1986. Before each set of experiments were initiated the six laboratory stocks were screened for DAV and DCV using the self-titration method. Partially purified preparations from self-titrated flies (see Section 5.2.1.2) were screened by immuno-osmoelectrophoresis. Before the commencement of the first set of experiments (August-November, 1985), each of the stocks produced only a single virus (as detectable by immuno-osmoelectrophoresis) after self-titration:

<table>
<thead>
<tr>
<th>Virus</th>
<th>Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAV</td>
<td>Cardwell (Mangoes)</td>
</tr>
<tr>
<td></td>
<td>Tamar Valley</td>
</tr>
<tr>
<td></td>
<td>Coffs Harbour</td>
</tr>
<tr>
<td>DCV</td>
<td>Ellis Beach</td>
</tr>
<tr>
<td></td>
<td>Araluen</td>
</tr>
<tr>
<td></td>
<td>Huonville</td>
</tr>
</tbody>
</table>
However, before the second group of experiments was started, a similar screening procedure revealed that most of the stocks contained DAV and DCV. Table 6.1 summarizes the reciprocal end-point dilutions (see Section 3.1.3.2), using immuno-osmoelectrophoresis, of each of the preparations produced after self-titration of these stocks.

**Table 6.1** Reciprocal end-point dilutions of self-titrated virus preparations produced after self-titration (June, 1986)

<table>
<thead>
<tr>
<th>Stock</th>
<th>DAV</th>
<th>DCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardwell (Mangoes) (CaM)</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>Ellis Beach (EB)</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>Coffs Harbour (HD)</td>
<td>32</td>
<td>1</td>
</tr>
<tr>
<td>Araluen (AN)</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>Tamar Valley (TR)</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>Huonville (HV)</td>
<td>32</td>
<td>-</td>
</tr>
</tbody>
</table>

Stocks were originally considered to be persistently infected with whichever virus was produced in the highest titre by self-titration. On the basis of such a classification the stocks can be divided as shown in Table 6.2.

**Table 6.2** Summary of laboratory stocks considered to be persistently infected with DAV and DCV during the two experimental periods of this study.

<table>
<thead>
<tr>
<th>Stock</th>
<th>1st Experimental Period</th>
<th>2nd Experimental Period</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAV</td>
<td>Cardwell (Mangoes) (CaM)</td>
<td>Cardwell (Mangoes)</td>
</tr>
<tr>
<td></td>
<td>Coffs Harbour (HD)</td>
<td>Coffs Harbour</td>
</tr>
<tr>
<td></td>
<td>Tamar Valley (TR)</td>
<td>Tamar Valley</td>
</tr>
<tr>
<td></td>
<td>Huonville (HV)</td>
<td>Huonville</td>
</tr>
<tr>
<td>DCV</td>
<td>Ellis Beach (EB)</td>
<td>Ellis Beach</td>
</tr>
<tr>
<td></td>
<td>Araluen</td>
<td>Araluen (AN)</td>
</tr>
</tbody>
</table>
However, using the results both from self-titration and the screening of individual flies from within cohorts (data below), shows that the situation is more complex and four qualitatively different infection states can be identified in the persistently infected *D. melanogaster* stocks (Table 6.3).

**Table 6.3** Classification of infection states identified in laboratory stocks of *D. melanogaster*

<table>
<thead>
<tr>
<th>Infection State</th>
<th>Self-Titration</th>
<th>In situ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>DAV&gt;DCV</td>
<td>DAV only</td>
</tr>
<tr>
<td>2.</td>
<td>DAV&gt;DCV</td>
<td>DAV and DCV</td>
</tr>
<tr>
<td>3.</td>
<td>DCV&gt;DAV</td>
<td>DCV and DAV</td>
</tr>
<tr>
<td>4.</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

For the purposes of the rest of this chapter, stocks showing the characteristics of infection state 1 will be referred to as "persistently infected with DAV", while those exhibiting group 3 characteristics will be referred to as "persistently DCV-infected". Obviously, those stocks exhibiting group 4 characteristics will be referred to as "virus-free". I should stress that, although lines are referred to as being persistently infected with either DAV or DCV, these terms really define the predominant virus within those stocks, and the division is based on the major qualitative differences between lines in the pattern in which the two viruses are expressed.

**6.3.2 Studies on Laboratory Stocks Persistently Infected with DAV.**

In the following sections reference will be made to the "frequency of infection of DAV". The detection sensitivity for DAV using the dot-blot assay described in Chapter 4 (see Figure 4.6) was found to be approximately $5 \times 10^7$ particles in isolates from single *Drosophila* (see
Section 4.3.4). In the studies reported in this chapter nearly all single *Drosophila* that were found to be positive for the presence of DAV were estimated to contain in the region of $1-3 \times 10^{10}$ particles. Therefore, "frequency of infection of DAV" refers not only to the proportion of flies in which DAV was detectable, but also to the level of detection, which was invariably in the range of $1-3 \times 10^{10}$ particles per fly.

6.3.2.1 Mortality

All relevant data from experiments in which mortality studies were carried out on laboratory stocks persistently infected with DAV are presented in Table 6.4. Considerable variation was found in mortality both within and between stocks (see Table 6.4). This variation was found to be associated with the four parameters listed below:

(i) differences between persistently DAV infected stocks derived from separate populations,

(ii) differences between cohorts within a stock,

(iii) differences between sexes in mortality,

(iv) differences arising after the virus(es) were removed from persistently infected stocks.

The results described below will focus on each of these areas in turn, and each area is subdivided to indicate where analyses were made in attempts to elucidate whether other factors had a role in determining mortality in the persistently DAV infected stocks.

The index of mortality that will be most commonly used in this section is $M_{24}$ - mortality at 24 days - a description of how this parameter is obtained is given in Section 6.2.1.1 (see also Figure 5.1B).
Table 6.4 Measured parameters for mortality experiments performed on the persistently DAV infected stocks Huonville, Tamar Valey, Coffs Harbour and Cardwell (Mangoes), during the periods August-November, 1985 (1st experimental period) and August-December, 1986 (2nd experimental period)

Days = the number of days that had elapsed between this replicate being set up and the start of the experimental period

\( T_{50} \) = time at which 50% of the total population had died

\( M_{24} \) = cumulative percentage mortality of the population at 24 days

\( N \) = number of flies used to initiate the experiment

WL = winglength, measured in graticule units (5 graticule units = 1mm); M = male, F = female

I.F.\( ^{DAV,30} \) = DAV infection frequency as a proportion of the total population surviving at 27-30 days

(B) = one fly found to contain both DAV and DCV

(+) = DAV detected in flies harvested prior to 30 days

I.F.\( ^{DCV,30} \) = DCV infection frequency as a proportion of the total population surviving at 27-30 days

(+) = DCV detected in flies harvested prior to 30 days

(0) = no virus detected

(C) = DCV detected in cadavers collected during the course of the experiment

(B) = one fly found to contain both DAV and DCV

NOTE: presence of virus in cadavers not indicated where positives were obtained for the virus at 27-30 days.
### Huonville (HV), 1\textsuperscript{st} experimental period.

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Days</th>
<th>N</th>
<th>$T_{50}$</th>
<th>$M_{24}$</th>
<th>I.F. (DAV,30)</th>
<th>I.F. (DCV,30)</th>
<th>Winglength</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>5</td>
<td>137</td>
<td>20.6</td>
<td>73</td>
<td>0.89</td>
<td>0.00</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>28</td>
<td>181</td>
<td>21.5</td>
<td>63</td>
<td>1.00</td>
<td>0.00</td>
<td>- 7.54</td>
</tr>
<tr>
<td>3.</td>
<td>28</td>
<td>140</td>
<td>30.4</td>
<td>62</td>
<td>-</td>
<td>-</td>
<td>- 7.54</td>
</tr>
<tr>
<td>4.</td>
<td>29</td>
<td>123</td>
<td>20.8</td>
<td>68</td>
<td>0.92</td>
<td>0.00</td>
<td>-</td>
</tr>
<tr>
<td>5.</td>
<td>33</td>
<td>136</td>
<td>17.0</td>
<td>73</td>
<td>1.00</td>
<td>0.00</td>
<td>6.89 7.75</td>
</tr>
<tr>
<td>6.</td>
<td>33</td>
<td>217</td>
<td>15.6</td>
<td>71</td>
<td>-</td>
<td>-</td>
<td>6.89 7.75</td>
</tr>
<tr>
<td>7.</td>
<td>35</td>
<td>100</td>
<td>23.0</td>
<td>56</td>
<td>1.00</td>
<td>0.00</td>
<td>- 7.21</td>
</tr>
<tr>
<td>8.</td>
<td>46</td>
<td>162</td>
<td>21.8</td>
<td>62</td>
<td>1.00</td>
<td>0.00</td>
<td>- 8.16</td>
</tr>
<tr>
<td>9.</td>
<td>46</td>
<td>205</td>
<td>26.6</td>
<td>36</td>
<td>-</td>
<td>-</td>
<td>- 8.16</td>
</tr>
<tr>
<td>10.</td>
<td>50</td>
<td>270</td>
<td>16.6</td>
<td>76</td>
<td>1.00</td>
<td>0.00</td>
<td>-</td>
</tr>
<tr>
<td>11.</td>
<td>63</td>
<td>167</td>
<td>25.5</td>
<td>44</td>
<td>0.75</td>
<td>0.00</td>
<td>- 8.05</td>
</tr>
<tr>
<td>12.</td>
<td>94</td>
<td>125</td>
<td></td>
<td>16</td>
<td>1.00</td>
<td>0.00</td>
<td>7.38 8.00</td>
</tr>
<tr>
<td>13</td>
<td>112</td>
<td>108</td>
<td>17.2</td>
<td>38</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

### Huonville, 2\textsuperscript{nd} experimental period

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Days</th>
<th>N</th>
<th>$T_{50}$</th>
<th>$M_{24}$</th>
<th>I.F. (DAV,30)</th>
<th>I.F. (DCV,30)</th>
<th>Winglength</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.</td>
<td>0</td>
<td>133</td>
<td>19.8</td>
<td>65</td>
<td>0.94</td>
<td>0.00</td>
<td>7.04 7.77</td>
</tr>
<tr>
<td>15.</td>
<td>7</td>
<td>101</td>
<td>18.4</td>
<td>58</td>
<td>1.00</td>
<td>0.00</td>
<td>6.64 7.46</td>
</tr>
<tr>
<td>16.</td>
<td>29</td>
<td>131</td>
<td>23.3</td>
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<td>0.00</td>
<td>- 7.80</td>
</tr>
<tr>
<td>17.</td>
<td>39</td>
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<td>20.0</td>
<td>70</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18.</td>
<td>39</td>
<td>199</td>
<td>28.6</td>
<td>35</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19.</td>
<td>44</td>
<td>177</td>
<td>21.1</td>
<td>65</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20.</td>
<td>111</td>
<td>221</td>
<td>14.6</td>
<td>75</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

### Tamar Valley (TR), 1\textsuperscript{st} experimental period

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Days</th>
<th>N</th>
<th>$T_{50}$</th>
<th>$M_{24}$</th>
<th>I.F. (DAV,30)</th>
<th>I.F. (DCV,30)</th>
<th>Winglength</th>
</tr>
</thead>
<tbody>
<tr>
<td>21.</td>
<td>28</td>
<td>199</td>
<td></td>
<td>27</td>
<td>0.98</td>
<td>0.00</td>
<td>6.87 7.72</td>
</tr>
<tr>
<td>22.</td>
<td>33</td>
<td>208</td>
<td>25.0</td>
<td>45</td>
<td>1.00</td>
<td>0.00</td>
<td>- 7.70</td>
</tr>
<tr>
<td>23.</td>
<td>33</td>
<td>203</td>
<td>26.2</td>
<td>48</td>
<td>-</td>
<td>-</td>
<td>- 7.70</td>
</tr>
<tr>
<td>24.</td>
<td>39</td>
<td>191</td>
<td></td>
<td>25</td>
<td>-</td>
<td>-</td>
<td>- 8.19</td>
</tr>
<tr>
<td>25.</td>
<td>61</td>
<td>82</td>
<td>35.4</td>
<td>43</td>
<td>1.00</td>
<td>0.00</td>
<td>- 7.96</td>
</tr>
</tbody>
</table>

### Tamar Valley, 2\textsuperscript{nd} experimental period

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Days</th>
<th>N</th>
<th>$T_{50}$</th>
<th>$M_{24}$</th>
<th>I.F. (DAV,30)</th>
<th>I.F. (DCV,30)</th>
<th>Winglength</th>
</tr>
</thead>
<tbody>
<tr>
<td>26.</td>
<td>0</td>
<td>132</td>
<td></td>
<td>24</td>
<td>0.96</td>
<td>0.00</td>
<td>6.49 6.99</td>
</tr>
<tr>
<td>27.</td>
<td>19</td>
<td>73</td>
<td></td>
<td>42</td>
<td>1.00</td>
<td>0.00</td>
<td>-</td>
</tr>
<tr>
<td>28.</td>
<td>19</td>
<td>227</td>
<td>20.2</td>
<td>61</td>
<td>-</td>
<td>-</td>
<td>- 7.73</td>
</tr>
<tr>
<td>29.</td>
<td>56</td>
<td>205</td>
<td></td>
<td>39</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>30.</td>
<td>108</td>
<td>139</td>
<td>23.1</td>
<td>53</td>
<td>1.00</td>
<td>0.00</td>
<td>6.52 7.46</td>
</tr>
</tbody>
</table>
### Coffs Harbour (HD), 1st experimental period

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Days</th>
<th>( N )</th>
<th>( T_{50} )</th>
<th>( M_{24} )</th>
<th>I.F. DAV, 30</th>
<th>I.F. DCV, 30</th>
<th>Wing length</th>
</tr>
</thead>
<tbody>
<tr>
<td>31.</td>
<td>5</td>
<td>141</td>
<td>-</td>
<td>22</td>
<td>1.00</td>
<td>0.00</td>
<td>-</td>
</tr>
<tr>
<td>32.</td>
<td>28</td>
<td>121</td>
<td>-</td>
<td>27</td>
<td>1.00</td>
<td>0.00</td>
<td>6.45</td>
</tr>
<tr>
<td>33.</td>
<td>28</td>
<td>128</td>
<td>23.7</td>
<td>52</td>
<td>-</td>
<td>-</td>
<td>6.45</td>
</tr>
<tr>
<td>34.</td>
<td>33</td>
<td>150</td>
<td>28.0</td>
<td>37</td>
<td>1.00</td>
<td>0.00</td>
<td>-</td>
</tr>
<tr>
<td>35.</td>
<td>33</td>
<td>148</td>
<td>21.3</td>
<td>60</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>36.</td>
<td>46</td>
<td>145</td>
<td>26.0</td>
<td>44</td>
<td>1.00</td>
<td>0.00</td>
<td>-</td>
</tr>
<tr>
<td>37.</td>
<td>50</td>
<td>142</td>
<td>-</td>
<td>19</td>
<td>0.96</td>
<td>0.00</td>
<td>-</td>
</tr>
<tr>
<td>38.</td>
<td>61</td>
<td>112</td>
<td>-</td>
<td>13</td>
<td>0.57</td>
<td>0.00</td>
<td>6.72</td>
</tr>
</tbody>
</table>

### Coffs Harbour, 2nd experimental period

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Days</th>
<th>( N )</th>
<th>( T_{50} )</th>
<th>( M_{24} )</th>
<th>I.F. DAV, 30</th>
<th>I.F. DCV, 30</th>
<th>Wing length</th>
</tr>
</thead>
<tbody>
<tr>
<td>39.</td>
<td>0</td>
<td>152</td>
<td>26.7</td>
<td>39</td>
<td>1.00</td>
<td>0.00</td>
<td>6.36</td>
</tr>
<tr>
<td>40.</td>
<td>7</td>
<td>224</td>
<td>29.2</td>
<td>33</td>
<td>0.92</td>
<td>0.00</td>
<td>5.96</td>
</tr>
<tr>
<td>41.</td>
<td>19</td>
<td>131</td>
<td>23.6</td>
<td>54</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>42.</td>
<td>19</td>
<td>202</td>
<td>22.4</td>
<td>54</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>43.</td>
<td>23</td>
<td>115</td>
<td>24.7</td>
<td>48</td>
<td>1.00</td>
<td>0.00</td>
<td>6.39</td>
</tr>
<tr>
<td>44.</td>
<td>39</td>
<td>70</td>
<td>-</td>
<td>15</td>
<td>1.00</td>
<td>0.00</td>
<td>-</td>
</tr>
<tr>
<td>45.</td>
<td>39</td>
<td>130</td>
<td>-</td>
<td>23</td>
<td>1.00</td>
<td>0.00</td>
<td>-</td>
</tr>
<tr>
<td>46.</td>
<td>39</td>
<td>255</td>
<td>27.0</td>
<td>46</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>47.</td>
<td>56</td>
<td>130</td>
<td>24.8</td>
<td>49</td>
<td>0.96</td>
<td>0.00</td>
<td>-</td>
</tr>
<tr>
<td>48.</td>
<td>108</td>
<td>121</td>
<td>19.7</td>
<td>58</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

### Cardwell (Mangoes) (CaM), 1st experimental period

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Days</th>
<th>( N )</th>
<th>( T_{50} )</th>
<th>( M_{24} )</th>
<th>I.F. DAV, 30</th>
<th>I.F. DCV, 30</th>
<th>Wing length</th>
</tr>
</thead>
<tbody>
<tr>
<td>49.</td>
<td>28</td>
<td>167</td>
<td>-</td>
<td>8</td>
<td>(+)</td>
<td>(0)</td>
<td>6.75</td>
</tr>
<tr>
<td>50.</td>
<td>33</td>
<td>154</td>
<td>23.5</td>
<td>53</td>
<td>1.00</td>
<td>0.00</td>
<td>-</td>
</tr>
<tr>
<td>51.</td>
<td>33</td>
<td>183</td>
<td>-</td>
<td>30</td>
<td>1.00</td>
<td>0.00</td>
<td>-</td>
</tr>
<tr>
<td>52.</td>
<td>39</td>
<td>122</td>
<td>-</td>
<td>5</td>
<td>1.00</td>
<td>0.00</td>
<td>-</td>
</tr>
<tr>
<td>53.</td>
<td>61</td>
<td>67</td>
<td>-</td>
<td>16</td>
<td>0.08</td>
<td>0.00</td>
<td>6.35</td>
</tr>
</tbody>
</table>

### Cardwell (Mangoes), 2nd experimental period

<table>
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<tr>
<th>Expt. No.</th>
<th>Days</th>
<th>( N )</th>
<th>( T_{50} )</th>
<th>( M_{24} )</th>
<th>I.F. DAV, 30</th>
<th>I.F. DCV, 30</th>
<th>Wing length</th>
</tr>
</thead>
<tbody>
<tr>
<td>54.</td>
<td>7</td>
<td>162</td>
<td>-</td>
<td>15</td>
<td>0.79</td>
<td>0.00</td>
<td>5.61</td>
</tr>
<tr>
<td>55.</td>
<td>19</td>
<td>140</td>
<td>-</td>
<td>9</td>
<td>0.00</td>
<td>0.00</td>
<td>6.20</td>
</tr>
<tr>
<td>56.</td>
<td>39</td>
<td>73</td>
<td>-</td>
<td>4</td>
<td>0.00</td>
<td>0.00</td>
<td>-</td>
</tr>
<tr>
<td>57.</td>
<td>39</td>
<td>127</td>
<td>-</td>
<td>6</td>
<td>0.00</td>
<td>0.00</td>
<td>-</td>
</tr>
<tr>
<td>58.</td>
<td>39</td>
<td>275</td>
<td>-</td>
<td>15</td>
<td>0.00</td>
<td>0.00</td>
<td>-</td>
</tr>
<tr>
<td>59.</td>
<td>44</td>
<td>151</td>
<td>-</td>
<td>12</td>
<td>1.00</td>
<td>0.00</td>
<td>-</td>
</tr>
<tr>
<td>60.</td>
<td>56</td>
<td>124</td>
<td>-</td>
<td>15</td>
<td>0.00</td>
<td>0.00</td>
<td>-</td>
</tr>
<tr>
<td>61.</td>
<td>111</td>
<td>228</td>
<td>-</td>
<td>39</td>
<td>1.00</td>
<td>0.00</td>
<td>-</td>
</tr>
</tbody>
</table>
### Huonville Virus-Free (HV<sub>ss</sub>), 2<sup>nd</sup> experimental period

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Days</th>
<th>N</th>
<th>T&lt;sub&gt;50&lt;/sub&gt;</th>
<th>M&lt;sub&gt;24&lt;/sub&gt;</th>
<th>I.F. DAV, 30</th>
<th>I.F. DCV, 30</th>
<th>M</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>62</td>
<td>0</td>
<td>120</td>
<td></td>
<td>8</td>
<td>0.00</td>
<td>0.00</td>
<td>6.89</td>
<td>7.64</td>
</tr>
<tr>
<td>63</td>
<td>18</td>
<td>111</td>
<td></td>
<td>4</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>28</td>
<td>202</td>
<td>25.9</td>
<td>40</td>
<td>1.00</td>
<td>0.00</td>
<td>7.35</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>45</td>
<td>194</td>
<td></td>
<td>31</td>
<td>0.94</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>118</td>
<td>162</td>
<td>25.7</td>
<td>45</td>
<td>1.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Coffs Harbour Virus-Free (HD<sub>ss</sub>), 2<sup>nd</sup> experimental period

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Days</th>
<th>N</th>
<th>T&lt;sub&gt;50&lt;/sub&gt;</th>
<th>M&lt;sub&gt;24&lt;/sub&gt;</th>
<th>I.F. DAV, 30</th>
<th>I.F. DCV, 30</th>
<th>M</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>67</td>
<td>0</td>
<td>172</td>
<td></td>
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<td>0.00</td>
<td>6.17</td>
<td>6.96</td>
</tr>
<tr>
<td>68</td>
<td>18</td>
<td>175</td>
<td></td>
<td>6</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>69</td>
<td>28</td>
<td>123</td>
<td></td>
<td>7</td>
<td>0.00</td>
<td>0.00</td>
<td>7.46</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>45</td>
<td>152</td>
<td></td>
<td>7</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>71</td>
<td>100</td>
<td>82</td>
<td></td>
<td>9</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
A. Population Differences in Mortality

All the mortality experiments were carried out during two periods; August - November, 1985 and August - December, 1986. Four laboratory populations were persistently infected with DAV in both these periods (see Section 6.3.1, Table 6.3); Cardwell (Mangoes) (CaM), Coffs Harbour (HD), Tamar Valley (TR) and Huonville (HV). As eight months elapsed between the two experimental periods, M$_{24}$ was compared within each population between the two experimental periods (Table 6.5). No significant difference was found between the mean M$_{24}$'s of any stock in the two periods. So, data from the two experimental periods were pooled for each stock. These pooled data will be used in all further analyses unless otherwise stated.

Comparisons of the mean M$_{24}$'s of the four laboratory stocks persistently infected with DAV, (Table 6.6 A and B) reveals considerable heterogeneity. The mean M$_{24}$ of each stock was compared to every other stock using the Student-Newman-Keuls multiple range test (Table 6.6C). The means can be grouped in the following way:

<table>
<thead>
<tr>
<th>HV</th>
<th>TR</th>
<th>HD</th>
<th>CaM</th>
</tr>
</thead>
</table>

It would appear that the populations with more southerly origins have greater mean mortality in laboratory culture than those from the more northerly populations. Whether this is associated with DAV cannot be established from these data. Nonetheless, variation in mortality both within and between these stocks could be accounted for by:

(i) variation within/between the stocks in resistance/susceptibility to the effects of DAV, or,

(ii) genetic variation in factors controlling life-expectancy within/between the stocks e.g. studies have shown that certain inbred lines of *D. melanogaster* have reduced viability (Pearl and Parker, 1922a) relative to the wild-type.
Table 6.5 Mean $M_{24}$ of Persistently DAV-Infected Laboratory stocks in Two Experimental Periods

<table>
<thead>
<tr>
<th></th>
<th>HV</th>
<th>TR</th>
<th>HD</th>
<th>Cam</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>56.31</td>
<td>37.64</td>
<td>34.25</td>
<td>24.42</td>
</tr>
<tr>
<td>SD</td>
<td>18.19</td>
<td>29.09</td>
<td>16.77</td>
<td>18.28</td>
</tr>
<tr>
<td>n</td>
<td>13</td>
<td>5</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td><strong>Group 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>59.86</td>
<td>43.82</td>
<td>41.91</td>
<td>14.38</td>
</tr>
<tr>
<td>SD</td>
<td>13.45</td>
<td>14.13</td>
<td>14.24</td>
<td>10.82</td>
</tr>
<tr>
<td>n</td>
<td>7</td>
<td>5</td>
<td>10</td>
<td>8</td>
</tr>
</tbody>
</table>

|                |     |     |     |     |
| F              | 1.832 | 4.234 | 1.387 | 2.847 |
| Between df     | [6, 12] | [4, 4] | [7, 9] | [4, 7] |
| Experimental Groups t | 0.452 | 0.430 | 1.052 | 1.252 |
| df             | [18] | [8]  | [16] | [11] |

(a) ; August-November, 1985
(b) ; August-December, 1986
(c) ; degrees of freedom
Table 6.6 Mean $M_{24}$ of laboratory stocks persistently infected with DAV and comparisons between these means

A. Mean $M_{24}$

B. Analysis of variance between means

C. q values obtained from each pairwise comparison of mean $M_{24}$ using the Student-Newman-Keuls test.
Table 6.6 Mean $M_{24}$ of persistently DAV-infected laboratory stocks, and comparisons between these means.

A.

<table>
<thead>
<tr>
<th></th>
<th>HV</th>
<th>TR</th>
<th>HD</th>
<th>CaM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>57.50</td>
<td>40.70</td>
<td>38.50</td>
<td>18.23</td>
</tr>
<tr>
<td>SD</td>
<td>16.40</td>
<td>12.88</td>
<td>15.44</td>
<td>14.34</td>
</tr>
<tr>
<td>n</td>
<td>20</td>
<td>10</td>
<td>18</td>
<td>13</td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>$F_{[3,53]}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock Means</td>
<td>12328.37</td>
<td>3</td>
<td>4109.46</td>
<td>18.03***</td>
</tr>
<tr>
<td>Error</td>
<td>12989.86</td>
<td>57</td>
<td>227.89</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>25318.23</td>
<td>60</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*** = p<0.001

C.

<table>
<thead>
<tr>
<th></th>
<th>HV</th>
<th>TR</th>
<th>HD</th>
<th>CaM</th>
</tr>
</thead>
<tbody>
<tr>
<td>HV</td>
<td>4.07*</td>
<td>4.27*</td>
<td>10.32*</td>
<td></td>
</tr>
<tr>
<td>TR</td>
<td>0.52</td>
<td>5.00*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HD</td>
<td></td>
<td>5.20*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* = p<0.05
Results presented in Chapter 5 indicated that after injection of DAV into *D. melanogaster* there was a delay between the virus reaching a high titre and the ensuing mortality (Section 5.3.1). In addition, the response of individual flies varied in the delay between the virus reaching a high titre and death. This observation suggests that within the stock tested (HD<sub>33</sub>), there is a factor responsible for conferring resistance to the pathogenic effect of DAV. Therefore, if the variation in M<sub>24</sub> between laboratory stocks is due to resistance factors against DAV it could be predicted that there will be variation in the frequency of DAV detection that is associated with the variation in mortality.

Cadavers collected during the experiments, together with the surviving flies, were screened for the presence of DAV and DCV. The frequency of DAV and DCV infection in these samples is summarized in Table 6.4.

Several features of these data are particularly interesting:

(i) in three of the stocks - Huonville (HV), Tamar Valley (TR) and Coffs Harbour (HD) - that were persistently infected with DAV in both experimental periods, DAV was always detected in flies that survived to the end of the experiment (27-30 days). The frequency of infection in these survivors was always in the range of 75-100%. Furthermore, despite the fact that DCV was produced during self-titration of Tamar Valley and Coffs Harbour flies in the second experimental period, DCV was never detected in either the survivors or the collected cadavers.

(ii) in the fourth of the laboratory stocks persistently infected with DAV in both experimental periods - Cardwell (Mangoes) - DAV was not detected in five out of 8 of the second group of experiments. Also, in one group of survivors the frequency of DAV infection was found to be only 8%. This was the lowest
frequency encountered at 30 days in any of the persistently
DAV infected lines. Therefore, it would seem that the
persistent DAV infection in this stock was either unstable
i.e. present in some cohorts but not in others, or that the
virus did not replicate in situ to a level detectable by the
assay being used i.e. titre/fly was less than $1 \times 10^7$ particles.
Despite the fact that DCV was produced after self-titration,
DCV was never detected in the survivors from the experiments
involving this stock or in cadavers collected during the
course of these experiments (see Section 6.3.1).

(iii) self-titration of the Araluen (AN) laboratory stock produced
mainly DAV before the second experimental period (see Section
6.3.1). In the first experimental period it produced only DCV
after self-titration. During the second experimental period
however, in contrast to those stocks that were persistently
infected with DAV in both periods, DCV was detected in flies
between the start (0 days) and termination (27-30 days) of
experiments (see, Section 6.3.2.2A). DCV was also detected in
cadavers collected during experiments involving this stock.
DAV still reached high frequencies of infection in this stock
at 27-30 days.

Contrary to its detection rate at 27-30 days, DAV was only rarely
detected in flies of 0-1 days old i.e. those flies used to initiate
mortality experiments. Therefore it would appear, given the high
frequency of infection at 27-30 days, that the frequency of infection
within a given cohort increases with time. This is supported by the
data presented in section 5.3.7. The variation in infection frequency
at 27-30 days could not however, explain the observed variation in
mortality. On the basis of the data presented so far, there would
appear to be no association between DAV and mortality.
B. Variation in Mortality Within Persistently DAV-Infected Stocks

In setting up the mortality experiments the numbers of flies were not strictly controlled because of the need to avoid any effects that anaesthesia might induce in infected flies. However, roughly similar numbers of flies (estimated by eye) were used for each replicate experiment. Pearl and Parker (1922b) found that for wild-type D. melanogaster the relationship between density and the duration of life was not linear. There was an optimal density either side of which the life-expectancy of the flies decreased. This optimal density was found to be approximately 40 flies in a half pint bottle. The size of the bottles used in Pearl and Parker’s study was approximately the same as the 250ml plastic bottles I used, so their results are probably applicable to the present study. If the shape of the curve of mortality on density is a skewed parabola, then a simple linear regression should approximate the curve at densities above the optimal. As the densities in all of the experiments discussed in this study were above the optimal of Pearl and Parker, the regression of \( M_{24} \) on density might be expected to be negative. Table 6.7 gives the results of simple regressions of \( M_{24} \) on density for each of the persistently DAV-infected laboratory stocks.

<table>
<thead>
<tr>
<th>HV</th>
<th>TR</th>
<th>HD</th>
<th>CaM</th>
</tr>
</thead>
<tbody>
<tr>
<td>( b^{(a)} )</td>
<td>0.064</td>
<td>0.025</td>
<td>0.102</td>
</tr>
<tr>
<td>( n )</td>
<td>20</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>( r^2 )</td>
<td>0.036</td>
<td>0.013</td>
<td>0.081</td>
</tr>
<tr>
<td>( p^{(b)} )</td>
<td>0.671</td>
<td>0.106</td>
<td>1.017</td>
</tr>
</tbody>
</table>

(a) \( b = \) simple regression coefficient

(b) \( degrees \ of \ freedom \ for \ F = [1,n-2] \)
None of the regressions of \( M_{24} \) on density are significantly different from 0. Furthermore, \( r^2 \) (the coefficient of determination for the regression; the value for \( r^2 \) is equivalent to the proportion of variation in the dependant variable explained by the regression) explains very little of the variation in any of the regressions. Therefore it would appear that over the range of densities used in the mortality experiments, density can only account for a small proportion of the observed variation in \( M_{24} \) in persistently DAV-infected stocks of \( D. \ melanogaster \). Inspection of the data does not suggest that other functions would fit the data better than a simple linear regression (Figure 6.1).

Another variable which might affect mortality within stocks is adult body size. Winglength was used as an index of adult body size in this study (see Section 6.2.3). Regressions of mortality (\( M_{24} \)) on female winglength were carried out to ascertain whether body size accounted for any of the variation in mortality within the persistently DAV-infected stocks. The results from these regressions are presented in Table 6.8

**Table 6.8** Simple regressions of \( M_{24} \) on female winglength in laboratory stocks persistently infected with DAV

<table>
<thead>
<tr>
<th></th>
<th>HD</th>
<th>TR</th>
<th>HD</th>
<th>CaM</th>
</tr>
</thead>
<tbody>
<tr>
<td>( b^{(a)} )</td>
<td>-20.10</td>
<td>2.48</td>
<td>9.91</td>
<td>-8.89</td>
</tr>
<tr>
<td>( n )</td>
<td>12</td>
<td>8</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>( r^2 )</td>
<td>0.154</td>
<td>0.004</td>
<td>0.033</td>
<td>0.045</td>
</tr>
<tr>
<td>( F^{(b)} )</td>
<td>1.539</td>
<td>0.024</td>
<td>0.394</td>
<td>0.492</td>
</tr>
</tbody>
</table>

(a) \( b = \) simple regression coefficient
(b) \( F = [1, n-2] \)

None of the regression slopes are significantly different from 0, and very little of the variation in \( M_{24} \) is explained by the
Figure 6.1 Cumulative percentage mortality at 24 days ($M_{24}$) plotted against starting density for the laboratory stocks Huonville (HV), Coffs Harbour (HD), Tamar Valley (TR) and Cardwell (Mangoes) (CaM).
Figure 6.1...continued

HD.

CaM.
regressions. Thus, it would appear that size, of which winglength is an index, is not associated with mortality/longevity in these four stocks. However, in the above analysis it was assumed that the relationship between winglength (size) and mortality would be linear. This need not be the case as there might be an optimal size for Drosophila with respect to mortality. If this were so then the shape of the curve of M_{24} on winglength would be parabolic. Inspection of the data does not suggest that any relationship other than linear would better fit the data (Figure 6.2).

In conclusion, it appears that neither infection frequency of DAV at 27-30 days nor the physical parameters - adult body size and density - can explain the variation in mortality (M_{24}) found within the four stocks investigated.

C. Sex Differences in Mortality

In the first set of experiments the mortality of males and females was often monitored separately. Therefore it was possible to measure M_{24} for males, females and for the whole cohort. These data are presented in Table 6.9. Sufficient data were available for comparisons of male and female mortality in the persistently DAV-infected stocks Huonville (HV) and Coffs Harbour (HD).

Significant correlations were found between male and female mortality (M_{24}) in both stocks (HV, r_{9} = 0.783, 0.01 > p > 0.005; HD, r_{4} = 0.944, 0.005 > p > 0.002). Male mortality was always lower than female mortality.

D. Mortality in Virus-Free Stocks

Previous studies have shown that removal of DPV and DCV from infected stocks is associated with a decrease in the endogenous mortality (Plus et al., 1975b; David and Plus, 1971). To ascertain
Figure 6.2 Cumulative percentage mortality at 24 days ($M_{24}$) plotted against wing length (WL) for the laboratory stocks Huonville (HV), Coffs Harbour (HD), Tamar Valley (TR) and Cardwell (Mangoes) (CaM).
Figure 6.2...continued

TR. C.P.M. (%)

40
20
60

Calm.

40
20
m 24

Wing length

Wing length
Table 6.9 Mortality parameters for males and females. All experiments were carried out during the first experimental period, August-November, 1985. Parameters and experimental numbers (No.) are as described in Table 6.4.

**Huonville (HV)**

<table>
<thead>
<tr>
<th>No.</th>
<th>N</th>
<th>T&lt;sub&gt;50&lt;/sub&gt;</th>
<th>M</th>
<th>F</th>
<th>M</th>
<th>F</th>
<th>M</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>69</td>
<td>54</td>
<td>28.0</td>
<td>16.0</td>
<td>45</td>
<td>93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>58</td>
<td>65</td>
<td>26.0</td>
<td>20.2</td>
<td>49</td>
<td>84</td>
<td></td>
<td></td>
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<tr>
<td>5</td>
<td>109</td>
<td>108</td>
<td>21.0</td>
<td>13.5</td>
<td>56</td>
<td>95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>64</td>
<td>72</td>
<td>28.0</td>
<td>13.5</td>
<td>45</td>
<td>99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>39</td>
<td>61</td>
<td>-</td>
<td>19.4</td>
<td>38</td>
<td>59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>79</td>
<td>83</td>
<td>-</td>
<td>18.4</td>
<td>43</td>
<td>87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>95</td>
<td>110</td>
<td>-</td>
<td>23.0</td>
<td>14</td>
<td>65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>135</td>
<td>135</td>
<td>20.4</td>
<td>15.6</td>
<td>63</td>
<td>95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>69</td>
<td>74</td>
<td>-</td>
<td>23.6</td>
<td>42</td>
<td>64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>53</td>
<td>72</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>53</td>
<td>55</td>
<td>-</td>
<td>22.8</td>
<td>23</td>
<td>54</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Tamar Valley (TR)**

<table>
<thead>
<tr>
<th>No.</th>
<th>N</th>
<th>T&lt;sub&gt;50&lt;/sub&gt;</th>
<th>M</th>
<th>F</th>
<th>M</th>
<th>F</th>
<th>M</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>101</td>
<td>102</td>
<td>29.3</td>
<td>20.8</td>
<td>28</td>
<td>71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>38</td>
<td>44</td>
<td>-</td>
<td>24.9</td>
<td>31</td>
<td>64</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Coffs Harbour (HD)**

<table>
<thead>
<tr>
<th>No.</th>
<th>N</th>
<th>T&lt;sub&gt;50&lt;/sub&gt;</th>
<th>M</th>
<th>F</th>
<th>M</th>
<th>F</th>
<th>M</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>65</td>
<td>61</td>
<td>-</td>
<td>20.7</td>
<td>26</td>
<td>99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>65</td>
<td>86</td>
<td>-</td>
<td>22.6</td>
<td>11</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>69</td>
<td>79</td>
<td>30.2</td>
<td>17.4</td>
<td>28</td>
<td>94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>59</td>
<td>66</td>
<td>30.8</td>
<td>20.0</td>
<td>20</td>
<td>76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>68</td>
<td>74</td>
<td>-</td>
<td>30.2</td>
<td>9</td>
<td>37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>63</td>
<td>53</td>
<td>-</td>
<td>-</td>
<td>8</td>
<td>21</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
whether removal of DAV from persistently infected stocks would lead to
a decrease in the mortality of the stock, virus-free stocks were
established as described in section 5.2.3, from the persistently DAV-
infected stocks Huonville (HV) and Coffs Harbour (HD). Data for the
mortality and physical parameters for the experiments involving these
virus-free stocks are summarized in Table 6.4.

The mean $M_{24}'s$ for the Huonville virus-free stock (HV$_{ss}$) and the
Coffs Harbour virus-free stock (HD$_{ss}$) are shown in Table 6.10.

Table 6.10 Mean $M_{24}$ in the Huonville (HV$_{ss}$) and Coffs Harbour (HD$_{ss}$)
 virus-free lines

<table>
<thead>
<tr>
<th></th>
<th>HV$_{ss}$</th>
<th>HD$_{ss}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>25.60</td>
<td>7.40</td>
</tr>
<tr>
<td>SD</td>
<td>18.64</td>
<td>1.14</td>
</tr>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

These data were compared with the data obtained using the relevant
persistently infected stock. A significant difference was found
between the mean $M_{24}$ of the Huonville virus-free stock and the
Huonville stock ($F_{[4,19]} = 1.29, t_{[23]} = 3.799, p < 0.001$). The variances of
the data from the Coffs Harbour virus-free stock and the Coffs Harbour
stock were found to be significantly different ($F_{[4,17]} = 183.80,
p < 0.001$), therefore the two samples were compared using the
nonparametric Mann-Whitney test. The samples from the two lines were
found to be significantly different ($U_{[5,19]} = 90, p < 0.001$).

To ascertain whether the virus-free lines were still virus-free,
the surviving flies at the termination of each experiment were
screened for the presence of DAV and DCV. Neither of these viruses
were detected in any of the flies from experiments using the Coffs
Harbour virus-free stock (HD$_{ss}$). However, DAV was detected in
survivors from the experiments with the Huonville virus-free stock (HVss). On inspection of the data from the different experiments using the HVss stock it appeared that after the start of the experiments there was a temporal pattern both in the virus infection frequency at 30 days and the observed M24 (Table 6.11).

Table 6.11 Temporal variation of M24 in the HVss stock and associated DAV infection frequencies

<table>
<thead>
<tr>
<th>DAYS</th>
<th>0</th>
<th>18</th>
<th>28</th>
<th>45</th>
<th>118</th>
</tr>
</thead>
<tbody>
<tr>
<td>M24</td>
<td>8</td>
<td>4</td>
<td>40</td>
<td>31</td>
<td>45</td>
</tr>
<tr>
<td>DAVIF,30</td>
<td>0.00</td>
<td>0.00</td>
<td>1.00</td>
<td>0.94</td>
<td>1.00</td>
</tr>
</tbody>
</table>

DAYS = days after commencement of experiments
DAVIF,30 = Infection frequency at 30 days

Both the Huonville and Coffs Harbour virus-free stocks had been maintained in the laboratory for 2 months prior to the commencement of the experiments. During this time they had been screened for the presence of viruses by the self-titration method (Section 3.1.2.2) and had been found to be virus-free. Therefore, from the above data it would appear that the Huonville virus-free stock became reinfected with DAV after the mortality experiments had been started. How this particular stock became re-infected is not known, but the re-infection appeared in the HVss stock despite the precautions that were taken to minimise the risk of virus contamination (see Section 2.3.2). It is interesting that of the 3 virus-free stocks established (HDss, EBss and HVss) HVss was the only stock that became re-infected, even though all three virus-free stocks were maintained under the same conditions i.e. in the same room and using the same culture techniques. Nevertheless, the reappearance of the virus in the HVss stock was
associated with an increase in the mortality of cohorts taken from the stock. This suggests that DAV infection does increase mortality.

In addition to these two virus-free stocks established from persistently DAV-infected laboratory stocks, the Cardwell (Mangoes) stock was found to have an "unstable" persistent DAV infection during the second experimental period (see Section 6.3.2.1A and Table 6.4). Although there was no difference between the mean $M_{24}$'s of this stock in the two experimental periods, inspection of the data reveals an apparent skewing of $M_{24}$ towards lower values in the experiments where DAV was not detected. The data from the experiments where DAV was detected were pooled, and compared with pooled data from experiments where DAV was absent (Table 6.12).

**Table 6.12** Mean $M_{24}$ in cohorts from the Cardwell (Mangoes) stock that were infected and uninfected with DAV

<table>
<thead>
<tr>
<th></th>
<th>DAV Infected</th>
<th>Uninfected</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
<td>23.50</td>
<td>9.80</td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td>15.97</td>
<td>5.07</td>
</tr>
<tr>
<td><strong>n</strong></td>
<td>8</td>
<td>5</td>
</tr>
</tbody>
</table>

The mortalities of these two samples were compared ($F_{[7, 4]} = 9.95, 0.05 > p > 0.02$; for samples with unequal variance Mann-Whitney test used, $U_{[5, 8]} = 35$, $0.05 > p > 0.025$). This demonstrates that lower mortalities occurred in experiments where DAV was not present. Mortality in the Coffs Harbour virus-free stock (HD$_{ss}$) and in the Cardwell (Mangoes) experiments where DAV was not detected did not differ statistically ($F_{[4, 4]} = 19.08$, $p < 0.01$, Mann-Whitney test, $U_{[5, 4]} = 14$, $p > 0.1$).

The data presented above show that:

(i) when DAV is a persistent infection it is associated with an increase in adult mortality ($M_{24}$),
(ii) in virus-free lines the endogenous mortality is approximately the same regardless of the origin of the stock. This contrasts with what is observed amongst persistently infected stocks in which DAV can be detected.

The second of the above observations suggests that there is variation in susceptibility to DAV amongst the persistently DAV-infected stocks. This difference in susceptibility might account for the differences in mean $M_{24}$ noted between the DAV infected stocks.

6.3.2.2 Temporal Variation in Cohorts

A cohort will be defined here as the aged progeny collected from routinely maintained laboratory cultures (see Section 3.2.2.1). Approximately fifty flies were removed from a 0-2 day old cohort, killed, and stored at $-20^\circ$C. The remainder of the cohort was divided into four approximately equal groups. Each group was placed onto fresh artificial diet and mortality was monitored as described in Section 6.2.1.1, with each group of flies being transferred to fresh diet every four days. Flies from one of the four replicates were harvested at 6, 12, 18 and 30 days, the flies frozen and stored at $-20^\circ$C. If insufficient flies were available to establish four bottles from a single cohort then three or two replicates were established and harvested at 6, 12 and 30 days, or 12 and 30 days respectively. Males and females from each group were screened individually for DAV and DCV as described in Section 5.2.4.

A. Virus Frequency

The frequencies of infected flies (males and females), at each of the times at which replicates were harvested, are shown in Table 6.13. It should be noted that DCV was detected along with DAV in experiments 47 and 48, in which the Araluen stock was used. Although three of the
Table 6.13 Temporal Variation in DAV infection frequency in cohorts from persistently DAV-infected laboratory stocks. Frequencies are given in percentages.

<table>
<thead>
<tr>
<th>Experiment (a)</th>
<th>Stock</th>
<th>0 M</th>
<th>0 F</th>
<th>6 M</th>
<th>6 F</th>
<th>12 M</th>
<th>12 F</th>
<th>18 M</th>
<th>18 F</th>
<th>30 M</th>
<th>30 F</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.</td>
<td>HV</td>
<td>0</td>
<td>0</td>
<td>25</td>
<td>42</td>
<td>81</td>
<td>83</td>
<td>94</td>
<td>92</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>15.</td>
<td>HV</td>
<td>0</td>
<td>0</td>
<td>33</td>
<td>38</td>
<td>54</td>
<td>94</td>
<td></td>
<td></td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>16.</td>
<td>HV</td>
<td>0</td>
<td>0</td>
<td>25</td>
<td>29</td>
<td>50</td>
<td>83</td>
<td></td>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>26.</td>
<td>TR</td>
<td>0</td>
<td>6</td>
<td>8</td>
<td>29</td>
<td>19</td>
<td>61</td>
<td>67</td>
<td>75</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>39.</td>
<td>HD</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td>11</td>
<td>17</td>
<td>36</td>
<td>59</td>
<td>71</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>40.</td>
<td>HD</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>31</td>
<td>33</td>
<td>38</td>
<td>40</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>43.</td>
<td>HD</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td>62</td>
<td>62</td>
<td></td>
<td></td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>54.</td>
<td>CaM</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>71</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>88.</td>
<td>AN</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td></td>
<td></td>
<td>28</td>
<td>61</td>
<td>97</td>
<td>91</td>
</tr>
<tr>
<td>89.</td>
<td>AN</td>
<td>-</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td></td>
<td></td>
<td>63</td>
<td>67</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>90.</td>
<td>AN</td>
<td>-</td>
<td>-</td>
<td>17 *</td>
<td>0 *</td>
<td>42 *</td>
<td>88</td>
<td>58 *</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

(a) - Experiment numbers are as designated in Table 6.4 and 6.20

* = DCV detected in these sample of flies along with DAV
other four lines considered to be persistently infected with DAV were found to produce DCV upon self-titration (Coffs Harbour, HD; Cardwell (Mangoes), CaM and Tamar Valley, TR), DCV was not detected in situ in any of these stocks (see Section 6.3.2.1A). As DCV was regularly detected in flies from the Araluen stock, and also in cadavers collected during experiments involving this stock, it was considered that the Araluen stock was not characteristic of the other persistently DAV-infected stocks (Section 6.3.1 - infection state 3, Table 6.3). Therefore, the results from this stock will not be included in the following analysis.

Within each cohort the general pattern was for the frequency of DAV infection to increase with time (Table 6.13). The infection frequency in males was consistently lower than in females, although there is no significant difference in the mean infection frequencies of males and females at any given time (6 days, $F_{[6,6]}=1.601$, $t_{[12]}=0.799$, 0.5>$p$>0.2; 12 days, $F_{[7,7]}=1.376$, $t_{[14]}=1.173$, 0.2>$p$>0.1; 18 days, $F_{[4,4]}=1.068$, $t_{[8]}=0.177$, 0.5>$p$). However, assuming that the distribution of skewed infection frequencies is the same in males and females then a $\chi^2$ analysis shows significant heterogeneity between the sexes ($\chi^2_{[1]}=5.67$, 0.025>$p$>0.01), implying that there tends to be a greater infection frequency in females.

As the number of cohorts that were tested from each persistently DAV-infected stock is low, all of the infection frequency data from the stocks Huonville (HV), Tamar Valley (TR), Coffs Harbour (HD) and Cardwell Mangoes (CaM) were pooled for further analysis. This procedure is based on the assumption that any effects that DAV might have are independent of the laboratory stock on which the observations have been made. That is, once the virus reaches a titre whereby it can be detected by the dot-blot assay (>4x10^{7} particles) any of the factors which may govern susceptibility or resistance to the virus play no further part.
Regressions were calculated of $M_{24}$ on the infection frequency of males and females at each of the times at which flies were harvested.

The results of these calculations are presented in Table 6.14.

**Table 6.14** Simple regressions of $M_{24}$ on male(M) and female(F) infection frequency

<table>
<thead>
<tr>
<th></th>
<th>6</th>
<th>12</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>F</td>
<td>M</td>
</tr>
<tr>
<td>$b$</td>
<td>1.230</td>
<td>0.856</td>
<td>0.586</td>
</tr>
<tr>
<td>$n$</td>
<td>7</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>$r^2$</td>
<td>0.801</td>
<td>0.618</td>
<td>0.859</td>
</tr>
<tr>
<td>$F(a)$</td>
<td>20.133**</td>
<td>8.072*</td>
<td>36.592***</td>
</tr>
</tbody>
</table>

*=p<0.05  **=p<0.01  ***=p<0.001

(a) - degrees of freedom for $F = [l, n-2]$

Significant regressions of $M_{24}$ are found both for male and female infection frequencies at 6 and 12 days, but not at 18 days. There is no significant difference in the slope of these regressions for males and females at either 6 or 12 days (6 days, $t_{[10]}=0.882, 0.5>p>0.2$; 12 days, $t_{[12]}=0.162, p>0.5$). There was no difference between the slopes of the regressions for females at 6 and 12 days ($t_{[11]}=1.332, p>0.5$), but the slopes of the regressions for males were significantly different ($t_{[11]}=2.407, 0.05>p>0.02$) at these times.

These data show that there is a significant association between the cumulative cohort mortality at 24 days ($M_{24}$) and infection frequency within the cohort at 6 and 12 days, but not at 18 days. If DAV is the causative agent of the mortality then there must be a delay between when the infection is detected and when it has its effect.

Given that the regressions of $M_{24}$ on infection frequencies at 6 and 12 days are significant, the implication is that the delay of DAV's putative mortality effect is in excess of 12 days. Regressions were
calculated of $M_{30}$ (mortality at 30 days) on infection frequency at 18
days to see if the delay was evident at later times in the cohorts
history. These regressions were not significant (males, $F_{[1, 3]}=9.44,$
$0.2>p>0.1$, $r^2=0.759$; females, $F_{[1, 3]}=7.66$, 0.2$p>0.1$, $r^2=0.719$)

Results presented in Chapter 5 (Section 5.3.7) suggested that the
infection frequencies observed prior to 12-15 days were a reflection
of the flies that were infected, via their mothers, upon eclosion.
Jousset and Plus (1975) showed that DAV-infected females stopped
transmitting virus to their progeny at about 15 days. Therefore, if
frequencies of infection up to this time are a reflection of the
emerging females that are persistently infected, and hence able to
transmit the virus in their gametes, any effect on their progeny
caused by vertical transmission of the virus should be strongest at
these times.

9 Progeny Sex-Ratio

Results presented previously have shown that mortality at 24 days,
and DAV infection frequencies at times up to 18 days post-eclosion, are
lower for males than they are for females. A lower infection frequency
in males up to 12 days old (i.e the proportion of flies infected at
elosion) could be explained in two ways;

(i) males acquire the virus less readily than females,

(ii) males that acquire the virus from their mothers or during
development are preferentially selected against before
eclosion.

The second of these explanations would lead to the prediction of a
sex-ratio skewed towards females in the progeny of DAV-infected
mothers. To test this the progenies arising from the cultures which
were harvested in order to score infection frequencies (Section
6.3.2.2A) were scored for the number of males and females. Flies were
collected for 10 days from the first emergence.
The data show that in all but two cases the number of male progeny was less than the number of female progeny (Table 6.15). This distribution of sex-ratios is significantly different from that expected under the null hypothesis of equality ($\chi^2_{[1]} = 5.82$, $0.025 > p > 0.01$), and shows there is a bias towards the production of female imagos.

Figure 6.3 summarizes the data in Table 6.15, and shows that there is a consistent pattern in the deviation from a 1:1 sex-ratio. To test whether there was any relationship between the infection frequency of mothers and the sex-ratio of their progeny, regressions of $\chi^2$ on female infection frequency were calculated. In addition, regressions of $\chi^2$ on larval density ($n$) and the winglength of mothers were calculated. The results are shown in Table 6.16.

The only significant regression found was between infection frequency at 12 days and the corresponding $\chi^2$ at this time. The regression of $\chi^2$ on infection frequency explained more of the variation in $\chi^2$ than did larval density, which explained very little of the variation in $\chi^2$.

Regressions of mean winglength of the mothers within a cohort explained almost 50% of the variation in $\chi^2$ at 6 and 12 days. This is surprising as mean female winglength remains effectively constant within a cohort throughout the experimental period tested in this study. In 12 experiments the winglength of females collected at the start and at the end of the experimental period was measured. In seven cases the mean winglength of these groups of females was not significantly different; in 2 cases it was significantly higher and in 3 cases significantly lower i.e. suggesting preferential mortality of smaller and larger flies respectively.

The above analysis suggests that there is some association between virus infection frequency of mothers up to 12 days and the sex-ratios
Table 6.15 $x^2$ values for the male:female ratio of progeny produced by mothers of different ages in persistently DAV-infected stocks

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Stock</th>
<th>Age of Mothers (days)</th>
<th>$x^2$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.</td>
<td>HV</td>
<td>6</td>
<td>7.51</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>10.62</td>
<td>544</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
<td>0.92</td>
<td>736</td>
</tr>
<tr>
<td>15.</td>
<td>HV</td>
<td>6</td>
<td>12.54</td>
<td>425</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>8.97</td>
<td>415</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>26.</td>
<td>TR</td>
<td>6</td>
<td>0.01*</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>9.90</td>
<td>481</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
<td>0.24</td>
<td>410</td>
</tr>
<tr>
<td>58.</td>
<td>HD</td>
<td>6</td>
<td>1.58</td>
<td>306</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>2.90</td>
<td>668</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
<td>0.22</td>
<td>456</td>
</tr>
<tr>
<td>59.</td>
<td>HD</td>
<td>6</td>
<td>2.17</td>
<td>310</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>3.68</td>
<td>393</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
<td>1.89</td>
<td>304</td>
</tr>
<tr>
<td>73.</td>
<td>CaM</td>
<td>6</td>
<td>0.02*</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>3.12</td>
<td>544</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
<td>1.31</td>
<td>736</td>
</tr>
</tbody>
</table>

$n$ = total number of progeny

* = number females > number of males
Figure 6.3 Deviation of progeny sex-ratios ($\chi^2$) produced by mothers of different ages in stocks persistently infected with DAV.

A. $\chi^2$

- $\times = \text{Expt. 14 (HV)}$
- $\ast = \text{Expt. 15 (HV)}$
- $\times = \text{Expt. 26 (TR)}$

B. $\chi^2$

- $\ast = \text{Expt. 39 (HD)}$
- $\circ = \text{Expt. 40 (HD)}$
- $\times = \text{Expt. 54 (CaM)}$
Table 6.16 Regressions of progeny sex-ratios on the infection frequency and winglength of their mothers and on larval density

<table>
<thead>
<tr>
<th>Age of Mothers (days)</th>
<th>6</th>
<th>12</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>6</th>
<th>12</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Infection</strong> b</td>
<td>0.201</td>
<td>0.090</td>
<td>0.012</td>
</tr>
<tr>
<td>r^2</td>
<td>0.517</td>
<td>0.736</td>
<td>0.343</td>
</tr>
<tr>
<td>r (a)</td>
<td>4.301</td>
<td>11.123*</td>
<td>-1.042</td>
</tr>
<tr>
<td><strong>Larval Density</strong> b</td>
<td>0.221</td>
<td>-0.014</td>
<td>-0.001</td>
</tr>
<tr>
<td>r^2</td>
<td>0.319</td>
<td>0.000</td>
<td>0.151</td>
</tr>
<tr>
<td>r (a)</td>
<td>1.869</td>
<td>8.4x10^-4</td>
<td>0.538</td>
</tr>
<tr>
<td><strong>Winglength</strong> b</td>
<td>7.470</td>
<td>5.273</td>
<td>-0.687</td>
</tr>
<tr>
<td>r^2</td>
<td>0.532</td>
<td>0.490</td>
<td>0.235</td>
</tr>
<tr>
<td>r (a)</td>
<td>4.367</td>
<td>1.882</td>
<td>0.937</td>
</tr>
</tbody>
</table>

*= 0.05>p>0.025

(a) - degrees of freedom for F = [1,n-2]
in their progeny. This topic requires more investigation as variation in other parameters e.g. winglength of mothers, can also affect the variation in the sex-ratio. Nonetheless, there is a consistent deficiency of male progeny produced by mothers up to 12 days in age, and it has previously been shown that females of greater than 15 days of age stop transmitting virus to their progeny via the cytoplasm of the egg (Jousset and Plus, 1975). This evidence strongly suggests that DAV passed from mothers to their progeny causes preferential mortality of pre-imago males.

6.3.2.3 Egg Production

In most studies on egg production in *D. melanogaster*, measurements have been made using single isolated females. In this study however, as the interactive effect between individuals within a cohort was considered to play an important role in determining the behaviour of the virus (e.g. see Section 5.3.7). Thus to detect any effects that the virus may have upon the flies measurements of egg production were made on groups of flies. From the total egg production at a given time and the number of females within the cohort, the average egg production per female was calculated. These data are summarized in Figure 6.4.

The patterns of egg production obtained in the current study (Figure 6.4B) are very different from those obtained in other studies where measurements were made on individual females (see, Figure 6.4A). The most noticeable differences are that in the present data peak production is skewed towards later times and fewer eggs are produced in each daily period (note, each point on the graphs in Figure 6.4 represents the average egg production per female in the previous 24 hours). Although these were common features of egg production in these data, there was considerable variation between groups of flies both in
Figure 6.4A "Standard" egg production-rate curve obtained in experiments where measurements of egg-production were made on single females. The figure below represents the average curve calculated from the data of Bouletreau-Merle et al., 1982; David et al., 1974; McMillan et al., 1970 and Partridge et al., 1986. D.E.P. = daily egg-production.
The numbers at the top corner of each graph are the experimental numbers presented in Table 6.4.
Figure 6.4B Daily egg production (D.E.P.) in stocks persistently infected with DAV.
Figure 6.4B. (continued)

8. D.E.P.

11. D.E.P.

12. D.E.P.

13. D.E.P.

(continued......)
Figure 6.4B .....continued

22. D.E.P.

0 4 8 12 16 20
Time (Days)

25. D.E.P.

0 4 8 12 16 20
Time (Days)

69. D.E.P.

0 4 8 12 16 20
Time (Days)

72. D.E.P.

0 4 8 12 16 20
Time (Days)

(continued.....)
Figure 6.4B. continued

52. D.E.P.

53. D.E.P.

55. D.E.P.

57. D.E.P.

Time (Days)

Time (Days)

Time (Days)

Time (Days)

The data for daily egg production were obtained by taking the mean of the two daily values for each egg production in a normal population containing 100 females at the commencement of the experiment. The data were calculated for each group of 10. To allow for death of females during the course of the experiment, the mid-point value of female mortality was used for each of the two day period. These data were summarized in Table 4.3a. The data from Townsville and Coffs Harbour were analysed by a two way analysis of variance. The results of these analyses are shown in Table 4.4a. This analysis reveals that there is no significant variation in either line between egg production in each two day period. This is somewhat surprising when the shape of the "standard" egg production pattern is considered. The data from each of the two figures, however, interactions between lines in egg production may have an important effect on the results of the experiment giving rise to local variation in egg production. Nevertheless, the analysis reveals that significant variation between groups in egg production is not likely to be present. Nevertheless, the analysis reveals that the results of the egg production in the egg production cohort was explained by temporal variation. The total egg production in the 4-20 day period was taken as an index of the egg production of each laboratory stock.
the magnitude of egg production rates and in the general shape of the curves.

Using the data for daily egg-production rate, two-daily values for total egg production in a nominal population containing 100 females (at the commencement of the experiment) were calculated for each group of data. To allow for death of females during the course of the experiment, the mid-point value of female mortality was used for each two day period. These data are summarized in Table 6.17.

The data from Huonville and Coffs Harbour were analysed by a two-way analysis of variance. The results of these analyses are shown in Table 6.18A and B. This analysis reveals that there is no significant variation in either line between egg production in each two day period. This is somewhat surprising when the shape of the "standard" egg production curve is considered (see Figure 6.4A). However, it should be remembered that the data dealt with here are for total cohorts and not for single representatives of those cohorts. Therefore, interactions between flies in a cohort may have an important effect on the results of the experiment giving rise to lower overall egg production for the cohort than would be predicted from single female studies. There also ensues a lack of temporal variation in egg production. Nevertheless, the analysis reveals highly significant variation between cohorts in egg production.

As so little of the total variation within the laboratory stocks was explained by temporal variation, the total egg production in the 4-20 day period was taken as an index of the egg production of each cohort. These data were then regressed on female mortality ($M_{24}$), winglength, and initial density. Only the data from the Huonville (HV) laboratory stock provided sufficient samples on which to calculate these regressions. The results are presented in Table 6.19.
Table 6.17 Egg production in two daily periods between 4 and 20 days in persistently DAV-infected stocks.

<table>
<thead>
<tr>
<th>Expt No.</th>
<th>Stock</th>
<th>4-6</th>
<th>6-8</th>
<th>8-10</th>
<th>10-12</th>
<th>12-14</th>
<th>14-16</th>
<th>16-18</th>
<th>18-20</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.</td>
<td>HV</td>
<td>1045</td>
<td>1010</td>
<td>914</td>
<td>878</td>
<td>1036</td>
<td>1081</td>
<td>819</td>
<td>569</td>
<td>7352</td>
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<tr>
<td>4.</td>
<td>HV</td>
<td>1448</td>
<td>1757</td>
<td>1777</td>
<td>1414</td>
<td>973</td>
<td>623</td>
<td>363</td>
<td>183</td>
<td>8538</td>
</tr>
<tr>
<td>5.</td>
<td>HV</td>
<td>724</td>
<td>651</td>
<td>799</td>
<td>1200</td>
<td>1238</td>
<td>1001</td>
<td>958</td>
<td>1155</td>
<td>7726</td>
</tr>
<tr>
<td>7.</td>
<td>HV</td>
<td>3208</td>
<td>3420</td>
<td>2880</td>
<td>2394</td>
<td>2091</td>
<td>1642</td>
<td>1185</td>
<td>883</td>
<td>17703</td>
</tr>
<tr>
<td>8.</td>
<td>HV</td>
<td>1085</td>
<td>1130</td>
<td>1170</td>
<td>1316</td>
<td>1583</td>
<td>1839</td>
<td>1857</td>
<td>1593</td>
<td>11575</td>
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<tr>
<td>11.</td>
<td>HV</td>
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<td>1931</td>
<td>2253</td>
<td>2151</td>
<td>2017</td>
<td>1864</td>
<td>1670</td>
<td>1340</td>
<td>14423</td>
</tr>
<tr>
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<td>2734</td>
<td>3506</td>
<td>3739</td>
<td>3552</td>
<td>3238</td>
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<td>2554</td>
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<td>24373</td>
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<td>23.</td>
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<td>1571</td>
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<td>2093</td>
<td>2089</td>
<td>1800</td>
<td>1547</td>
<td>1245</td>
<td>980</td>
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<td>33.</td>
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<td>864</td>
<td>957</td>
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<td>1371</td>
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<td>9002</td>
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<td>1776</td>
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<td>1726</td>
<td>1856</td>
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<td>HD</td>
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<td>1585</td>
<td>2439</td>
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<td>3190</td>
<td>2789</td>
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<td>19457</td>
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<td>38.</td>
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<td>637</td>
<td>1661</td>
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<td>2399</td>
<td>2567</td>
<td>2769</td>
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<td>50.</td>
<td>CaM</td>
<td>1580</td>
<td>2019</td>
<td>2233</td>
<td>2175</td>
<td>2004</td>
<td>1870</td>
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<td>14887</td>
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<td>53.</td>
<td>CaM</td>
<td>700</td>
<td>900</td>
<td>1090</td>
<td>1100</td>
<td>1099</td>
<td>1109</td>
<td>1119</td>
<td>1079</td>
<td>8196</td>
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</tbody>
</table>
Table 6.18 Analysis of Variance of egg production between 4 and 20 days in Huonville and Coffs Harbour Laboratory stocks

A. Huonville (HV)

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS(x10^-7)</th>
<th>df</th>
<th>MS</th>
<th>p (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td>0.4572</td>
<td>7</td>
<td>0.0653</td>
<td>1.666</td>
</tr>
<tr>
<td>Experiments</td>
<td>3.2276</td>
<td>7</td>
<td>0.4611</td>
<td>11.763***</td>
</tr>
<tr>
<td>Residual</td>
<td>1.9254</td>
<td>49</td>
<td>0.0392</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5.6103</td>
<td>63</td>
<td>0.0891</td>
<td></td>
</tr>
</tbody>
</table>

B. Coffs Harbour (HD)

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS(x10^-7)</th>
<th>df</th>
<th>MS</th>
<th>p (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td>0.1468</td>
<td>7</td>
<td>0.0210</td>
<td>0.798</td>
</tr>
<tr>
<td>Experiments</td>
<td>0.8273</td>
<td>3</td>
<td>0.2763</td>
<td>10.506***</td>
</tr>
<tr>
<td>Residual</td>
<td>0.5527</td>
<td>21</td>
<td>0.0263</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1.5323</td>
<td>31</td>
<td>0.0494</td>
<td></td>
</tr>
</tbody>
</table>

(a) - degrees of freedom for F = [df Days/Experiments, df Residual]  
*** = p<0.001
Table 6.19 Simple regressions of total egg production on female mortality ($M_{24}$), winglength (WL) and initial density (D) in the persistently DAV-infected Huonville (HV) Laboratory stock

<table>
<thead>
<tr>
<th></th>
<th>$M_{24}$</th>
<th>N</th>
<th>WL</th>
</tr>
</thead>
<tbody>
<tr>
<td>b</td>
<td>-261.62</td>
<td>78.91</td>
<td>1992.90</td>
</tr>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>$r^2$</td>
<td>0.945</td>
<td>0.053</td>
<td>0.011</td>
</tr>
<tr>
<td>$F(a)$</td>
<td>103.889***</td>
<td>0.366</td>
<td>$8 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

*** = $p<0.001$

(a) - degrees of freedom for $F = [1, n-2]$

Only the negative regression of total egg production on mortality was significant. This regression explains 95% of the variation in total egg production.

6.3.3 Studies on Laboratory Stocks Persistently Infected with DCV

6.3.3.1 Mortality

Two lines were found to be persistently infected with DCV before the first experimental period (August-November, 1985) - Ellis Beach and Araluen. By the commencement of the second experimental period the Araluen stock had changed in its infection state (Table 6.3) and although it produced mostly DAV after self-titration (see Section 6.3.1) DCV was also detectable in situ. The mortality data for these two lines are presented in Table 6.20.

A. Temporal and Inter-Stock Variation in Mortality

The data in Table 6.20 show that during the first experimental period, both the Ellis Beach and the Araluen stocks went through a short period when mortality was greater than at all other times i.e
Table 6.20 Measured parameters for mortality experiments performed on the persistently DCV infected stocks Araluen and Ellis Beach, during the periods August-November, 1985 (1st experimental period) and August-December, 1986 (2nd experimental period)

Days = the number of days that had elapsed between this replicate being set up and the start of the experimental period

$T_{50}$ = time at which 50% of the total population had died

$M_{24}$ = cumulative percentage mortality of the population at 24 days

$N$ = number of flies used to initiate the experiment

$WL$ = winglength, measured in graticule units (5 graticule units = 1 mm); $M$ = male, $F$ = female

$I.F.\text{DAV, }30$ = DAV infection frequency as a proportion of the total population surviving at 27-30 days

(B) = one fly found to contain both DAV and DCV

(+) = DAV detected in flies harvested prior to 30 days

$I.F.\text{DCV, }30$ = DCV infection frequency as a proportion of the total population surviving at 27-30 days

(+) = DCV detected in flies harvested prior to 30 days

(0) = no virus detected

(C) = DCV detected in cadavers collected during the course of the experiment

(B) = one fly found to contain both DAV and DCV

NOTE: presence of virus in cadavers not indicated where positives were obtained for the virus at 27-30 days.
### Araluen, 1st experimental period

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Days</th>
<th>D</th>
<th>T_{50}</th>
<th>M_{24}</th>
<th>I.F._{DAV,30}</th>
<th>I.F._{DCV,30}</th>
<th>M</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>72.</td>
<td>5</td>
<td>84</td>
<td>-</td>
<td>34</td>
<td>0.00</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>73.</td>
<td>28</td>
<td>202</td>
<td>5.6</td>
<td>85</td>
<td>1.00</td>
<td>0.00</td>
<td>6.01</td>
<td>6.90</td>
</tr>
<tr>
<td>74.</td>
<td>28</td>
<td>250</td>
<td>3.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>75.</td>
<td>33</td>
<td>276</td>
<td>4.0</td>
<td>-</td>
<td>0.00</td>
<td>0.23</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>76.</td>
<td>35</td>
<td>401</td>
<td>5.6</td>
<td>73</td>
<td>0.89 (B)</td>
<td>0.11 (B)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>78.</td>
<td>53</td>
<td>196</td>
<td>26.6</td>
<td>46</td>
<td>0.00</td>
<td>0.07</td>
<td>7.20</td>
<td></td>
</tr>
<tr>
<td>79.</td>
<td>53</td>
<td>291</td>
<td>24.4</td>
<td>51</td>
<td>-</td>
<td>-</td>
<td>7.20</td>
<td></td>
</tr>
<tr>
<td>80.</td>
<td>63</td>
<td>221</td>
<td>-</td>
<td>33</td>
<td>0.00</td>
<td>0.02</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>81.</td>
<td>63</td>
<td>165</td>
<td>-</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>7.02</td>
<td></td>
</tr>
<tr>
<td>82.</td>
<td>63</td>
<td>262</td>
<td>27.6</td>
<td>29</td>
<td>-</td>
<td>-</td>
<td>7.02</td>
<td></td>
</tr>
<tr>
<td>83.</td>
<td>73</td>
<td>85</td>
<td>-</td>
<td>23</td>
<td>0.00</td>
<td>0.07</td>
<td>7.42</td>
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</tr>
<tr>
<td>84.</td>
<td>81</td>
<td>90</td>
<td>25.1</td>
<td>42</td>
<td>0.38</td>
<td>0.08</td>
<td>7.40</td>
<td></td>
</tr>
<tr>
<td>85.</td>
<td>94</td>
<td>44</td>
<td>-</td>
<td>14</td>
<td>-</td>
<td>-</td>
<td>6.62</td>
<td>7.35</td>
</tr>
<tr>
<td>86.</td>
<td>95</td>
<td>119</td>
<td>-</td>
<td>31</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>87.</td>
<td>104</td>
<td>125</td>
<td>25.6</td>
<td>44</td>
<td>0.26</td>
<td>0.16</td>
<td>7.35</td>
<td></td>
</tr>
</tbody>
</table>

### Araluen, 2nd experimental period

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Days</th>
<th>D</th>
<th>T_{50}</th>
<th>M_{24}</th>
<th>I.F._{DAV,30}</th>
<th>I.F._{DCV,30}</th>
<th>M</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>88.</td>
<td>7</td>
<td>151</td>
<td>-</td>
<td>36</td>
<td>0.95</td>
<td>(c)</td>
<td>6.30</td>
<td>6.91</td>
</tr>
<tr>
<td>89.</td>
<td>19</td>
<td>157</td>
<td>-</td>
<td>39</td>
<td>0.95</td>
<td>(d)</td>
<td>6.46</td>
<td>7.11</td>
</tr>
<tr>
<td>90.</td>
<td>43</td>
<td>131</td>
<td>-</td>
<td>46</td>
<td>1.00</td>
<td>(d)</td>
<td>-</td>
<td>7.31</td>
</tr>
<tr>
<td>91.</td>
<td>111</td>
<td>197</td>
<td>12.8</td>
<td>70</td>
<td>0.96</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

### Ellis Beach (EB), 1st experimental period

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Days</th>
<th>D</th>
<th>T_{50}</th>
<th>M_{24}</th>
<th>I.F._{DAV,30}</th>
<th>I.F._{DCV,30}</th>
<th>M</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>92.</td>
<td>5</td>
<td>135</td>
<td>-</td>
<td>45</td>
<td>0.00</td>
<td>0.04</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>93.</td>
<td>18</td>
<td>215</td>
<td>16.8</td>
<td>60</td>
<td>1.00 (B)</td>
<td>0.04 (B)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>94.</td>
<td>28</td>
<td>243</td>
<td>16.4</td>
<td>65</td>
<td>-</td>
<td>(+)</td>
<td>6.17</td>
<td>7.03</td>
</tr>
<tr>
<td>95.</td>
<td>29</td>
<td>195</td>
<td>6.0</td>
<td>-</td>
<td>0.00</td>
<td>0.35</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>96.</td>
<td>33</td>
<td>260</td>
<td>6.6</td>
<td>-</td>
<td>0.00</td>
<td>1.00</td>
<td>6.28</td>
<td>7.12</td>
</tr>
<tr>
<td>97.</td>
<td>33</td>
<td>137</td>
<td>5.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6.28</td>
<td>7.12</td>
</tr>
<tr>
<td>98.</td>
<td>35</td>
<td>124</td>
<td>29.0</td>
<td>34</td>
<td>0.89</td>
<td>0.03</td>
<td>-</td>
<td>6.64</td>
</tr>
<tr>
<td>99.</td>
<td>39</td>
<td>171</td>
<td>24.0</td>
<td>50</td>
<td>1.00</td>
<td>(c)</td>
<td>-</td>
<td>7.79</td>
</tr>
<tr>
<td>100.</td>
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<td>119</td>
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<td>0.00</td>
<td>-</td>
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<tr>
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<td>-</td>
<td>21</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>102.</td>
<td>94</td>
<td>78</td>
<td>-</td>
<td>14</td>
<td>0.33</td>
<td>0.00</td>
<td>6.62</td>
<td>7.35</td>
</tr>
<tr>
<td>103.</td>
<td>96</td>
<td>126</td>
<td>31</td>
<td>1.00</td>
<td>(c)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>104.</td>
<td>96</td>
<td>40</td>
<td>-</td>
<td>30</td>
<td>0.00</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>105.</td>
<td>112</td>
<td>114</td>
<td>24.2</td>
<td>48</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Expt. No.</td>
<td>Days</td>
<td>EBF</td>
<td>D</td>
<td>T$_{50}$</td>
<td>M$_{24}$</td>
<td>I.F. DAV, 30</td>
<td>I.F. DCV, 30</td>
<td>Winglength</td>
</tr>
<tr>
<td>-----------</td>
<td>------</td>
<td>-----</td>
<td>---</td>
<td>---------</td>
<td>-------</td>
<td>-------------</td>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Ellis Beach, 2$^{nd}$ experimental period</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>106.</td>
<td>15</td>
<td>141</td>
<td>-</td>
<td>40</td>
<td>0.00</td>
<td>0.06</td>
<td>-</td>
<td>6.48</td>
</tr>
<tr>
<td>107.</td>
<td>19</td>
<td>126</td>
<td>-</td>
<td>32</td>
<td>0.00</td>
<td>0.04</td>
<td>6.27</td>
<td>7.15</td>
</tr>
<tr>
<td>108.</td>
<td>23</td>
<td>46</td>
<td>23.5</td>
<td>52</td>
<td>1.00</td>
<td>(C)</td>
<td>6.31</td>
<td>7.28</td>
</tr>
<tr>
<td>109.</td>
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<td>136</td>
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<td>53</td>
<td>0.84</td>
<td>(C)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>110.</td>
<td>23</td>
<td>239</td>
<td>16.5</td>
<td>64</td>
<td>0.81</td>
<td>(C)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>111.</td>
<td>29</td>
<td>116</td>
<td>26.6</td>
<td>45</td>
<td>0.00</td>
<td>0.11</td>
<td>-</td>
<td>6.92</td>
</tr>
<tr>
<td>112.</td>
<td>39</td>
<td>59</td>
<td>-</td>
<td>7</td>
<td>0.00</td>
<td>0.02</td>
<td>-</td>
<td>7.26</td>
</tr>
<tr>
<td>113.</td>
<td>39</td>
<td>132</td>
<td>-</td>
<td>11</td>
<td>0.00</td>
<td>(C)</td>
<td>-</td>
<td>7.26</td>
</tr>
<tr>
<td>114.</td>
<td>39</td>
<td>234</td>
<td>-</td>
<td>24</td>
<td>0.00</td>
<td>0.00</td>
<td>-</td>
<td>7.26</td>
</tr>
<tr>
<td>115.</td>
<td>43</td>
<td>202</td>
<td>-</td>
<td>31</td>
<td>-</td>
<td>(C)</td>
<td>-</td>
<td>7.26</td>
</tr>
<tr>
<td>116.</td>
<td>56</td>
<td>153</td>
<td>-</td>
<td>16</td>
<td>0.00</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>117.</td>
<td>108</td>
<td>194</td>
<td>-</td>
<td>23</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
<td>6.64</td>
</tr>
<tr>
<td>118.</td>
<td>108</td>
<td>108</td>
<td>-</td>
<td>21</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ellis Beach Virus-Free (EB$_{ss}$), 2$^{nd}$ experimental period</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>119.</td>
<td>0</td>
<td>144</td>
<td>-</td>
<td>13</td>
<td>0.00</td>
<td>0.00</td>
<td>6.56</td>
<td>7.26</td>
</tr>
<tr>
<td>120.</td>
<td>28</td>
<td>181</td>
<td>-</td>
<td>4</td>
<td>0.00</td>
<td>0.00</td>
<td>-</td>
<td>7.69</td>
</tr>
<tr>
<td>121.</td>
<td>45</td>
<td>125</td>
<td>-</td>
<td>4</td>
<td>0.00</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>122.</td>
<td>100</td>
<td>143</td>
<td>-</td>
<td>6</td>
<td>0.00</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>123.</td>
<td>100</td>
<td>87</td>
<td>-</td>
<td>9</td>
<td>0.00</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
T<sub>50</sub> was below 10 days while the lowest T<sub>50</sub> noted in any other experiment (including DAV- and DCV-persistently infected lines) was 14.6 days and T<sub>50</sub> was generally in excess of 20 days. Figure 6.5 summarizes the temporal variation in T<sub>50</sub> during the first experimental period both for the Ellis Beach and Araluen stocks.

As can be seen in Figure 6.5, the distribution of high mortality (low T<sub>50</sub>) through time is not uniform. The experiments can therefore be divided into two groups on the basis of the temporal variation in mortality, one group up to when T<sub>50</sub> was at its minimum, and a second group after this point. These periods will be termed 1A and 1B respectively. Mortality in these two periods was compared using the Mann-Whitney test (as T<sub>50</sub> was <10 days, no value of M<sub>24</sub> was generally calculable to allow for a parametric test). In both stocks, the two periods were found to have statistically different mortalities (Araluen, U<sub>[5,14]</sub>=46, p=0.01; Ellis Beach, U<sub>[6,8]</sub>=52, p<0.001).

The Ellis Beach stock was classified as being persistently infected with DCV in both experimental periods (1 and 2 - see Section 6.3.1). Therefore, the mortality in group 1A was compared with that in experimental period 2. The two mortalities were found to be significantly different. Mortalities in experimental period 1B and experimental period 2 were then compared (Table 6.21). No significant difference was found in the mean M<sub>24</sub> of these two periods. These two groups of data (Ellis Beach 1B and experimental period 2) were then pooled and compared with those of the Araluen stock (1B). No significant difference was found between these means (Table 6.21). Therefore, mean mortalities in the Ellis Beach and Araluen stocks, after times of high mortality in the first experimental period, were homogenous.

The survivors in most of the experiments involving DCV infected laboratory stocks were screened for DCV and DAV. Four types of
Figure 6.5 Temporal variation of T50 in Ellis Beach (o—o) and Araluen (x—x) laboratory stocks during the period August-November, 1985. d = time (days) after the first experiment in this period was performed.
Table 6.21 Comparison of mean $M_{24}$ between experimental groups of the Ellis Beach stock (A), and between experimental periods 1B and 2 of the Ellis Beach stock and 1B of the Araluen stock (B)

<table>
<thead>
<tr>
<th></th>
<th>Group 1B</th>
<th>Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>30.89</td>
<td>32.32</td>
</tr>
<tr>
<td>SD</td>
<td>11.93</td>
<td>17.53</td>
</tr>
<tr>
<td>n</td>
<td>9</td>
<td>13</td>
</tr>
</tbody>
</table>

$F_{[8,12]} = 2.16, t_{[20]} = 0.212, p > 0.5$

<table>
<thead>
<tr>
<th></th>
<th>Ellis B.</th>
<th>Araluen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>31.68</td>
<td>34.50</td>
</tr>
<tr>
<td>SD</td>
<td>15.17</td>
<td>11.32</td>
</tr>
<tr>
<td>n</td>
<td>22</td>
<td>10</td>
</tr>
</tbody>
</table>

$F_{[9,21]} = 1.794, t_{[30]} = 0.504, p > 0.5$
surviving groups could be identified on the basis of the viruses that were detected:

(i) only DCV was present
(ii) only DAV was present
(iii) DAV and DCV were present
(iv) no viruses could be detected.

Each experiment using the DCV-infected lines was classified into one of the above four groups and the mean $M_{24}$ calculated for each of these groups (only data from groups 1B of both stocks and group 2 of the Ellis beach stock were used in this analysis). An analysis of variance of these data revealed no significant heterogeneity in the four groups. Therefore, it seemed that little of the variation in $M_{24}$ within these groups could be explained by qualitative variation in the virus(es) present within the individual cohorts. However, this lack of variation in the mean $M_{24}$ of the four groups must be considered in relation to five observations:

(i) even though, in the experimental periods under study, Ellis Beach and Araluen were considered to be persistently infected with DCV (see Section 3.5.1), DAV was regularly detected,

(ii) the frequency of DAV infection in the persistently DCV-infected stocks was lower than in persistently DAV-infected stocks when DCV was known to be present i.e. detected in cadavers or survivors (see Tables 6.20 and 6.4). This difference in detection frequency was significantly different from that found in DAV infected stocks - which also produced DCV after self-titration - $F_{[11,29]}=7.27$, $p<0.001$; Mann-Whitney test used for samples with unequal variance, $U_{[12,30]}=176$, $0.05>p>0.02$,

(iii) the frequency of DCV detection was very low compared to that of DAV in most groups of survivors,
(iv) in many experiments DCV was detected in collected cadavers even though it was not detected in flies surviving to the termination of the experiment,

(v) DAV and DCV were detected together in some groups of flies, and within these groups individual flies were occasionally found that had detectable levels of both viruses.

These observations suggest that there is a complex interaction between DAV and DCV in some laboratory stocks that leads to the suppression - at least in part - of DAV. What is perhaps more interesting is that the effect appears to be limited to certain stocks. For instance, it is known from the self-titration studies that DCV was present in the Tamar Valley, Coffs Harbour and Cardwell (Mangoes) stocks before the second series of experiments commenced. However, the virus was never detected in situ in flies from these stocks. In contrast, the Araluen and Ellis Beach stocks, which contained DCV, had depressed levels of DAV and regularly tested positive for DCV in situ. Therefore, although both viruses were present in most of the laboratory stocks under investigation, there were considerable qualitative differences between the stocks.

A further observation that might bear upon this problem is that both the Ellis Beach and Araluen stocks went through a "bottle-neck" of high mortality in the first experimental period. It has previously been reported that laboratory stocks persistently infected with DCV can have high endogenous mortality, with in some cases, most flies dying within 8 days of eclosion (Plus et al., 1975b). Given this fact, the high mortality in the Ellis Beach and Araluen stocks might be due to DCV, and DAV may have appeared as a later infection. If this is what happened then there should be a temporal pattern to the presence of viruses within the two stocks i.e. DCV followed by DCV and DAV followed by DAV alone. Using the Ellis Beach data (experimental
periods 1 and 2), the distribution through time of the four different groups of survivors, characterised by the presence/absence of DCV and DAV, was tested using the Kruskal-Wallis test. This analysis revealed no significant difference in the temporal distribution of the four virus-typed groups ($H=5.39$, $k=4$ so the $\chi^2$ approximation used for $H$, $0.25>p>0.1$). Thus, it would seem that there is no evidence for a temporal sequence in the distribution of the four virus-typed groups.

B. Mortality in Virus-Free Stocks

A virus-free line of flies (EB$_{ss}$) was established from the Ellis Beach stock (see Section 6.2.3). These flies were screened by self-titration and direct dot-blot assay of surviving flies (27-30 days old) from the mortality experiments. Neither DAV nor DCV were detected in any of the mortality experiments using this stock. The mean $M_{24}$ of this stock was compared with that of its parental stock (EB) (Table 6.22).

<table>
<thead>
<tr>
<th>Table 6.22</th>
<th>Mean $M_{24}$ for Ellis Beach stock (EB) and the virus-free stock established from it (EB$_{ss}$).</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EB</td>
</tr>
<tr>
<td>Mean</td>
<td>31.68</td>
</tr>
<tr>
<td>SD</td>
<td>15.17</td>
</tr>
<tr>
<td>n</td>
<td>22</td>
</tr>
</tbody>
</table>

The mortalities in these two groups were found to be significantly different ($F_{[21,4]}=15.66$, $0.05>p>0.02$; Mann-Whitney test for samples with unequal variance, $U_{[5,22]}=107$, $p<0.001$). This result demonstrates that removal of the virus(es) from the Ellis Beach stock appears to lead to a decrease in the endogenous mortality of the stock.
C. Temporal Variation Within Cohorts

In laboratory stocks persistently infected with DAV considerable temporal variation was found in the frequency of DAV infection within single cohorts (see Section 6.3.2.2A). This variation could have been predicted from the high frequency of infection found at 27-30 days. No similar level of infection was found for DCV at 27-30 days. Therefore, the question arises: why is the infection frequency of DCV so low at these times in comparison to that found for DAV? Two possibilities can be considered:

(i) either the time-infection frequency curve is parabolic i.e. reaches a high frequency before 27-30 days and then falls to the level detected; or,

(ii) the infection frequency remains low throughout time within the cohort.

To test these possibilities cohorts from the Ellis Beach stock were screened for DCV at various times as described in Section 6.3.2.2. The results are shown in Table 6.23.

Table 6.23 Temporal variation in the frequency of DCV detection in cohorts from the Ellis Beach laboratory stock.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Day</th>
<th>0</th>
<th>6</th>
<th>12</th>
<th>18</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>106.</td>
<td></td>
<td>0.00</td>
<td>0.05</td>
<td>0.02</td>
<td>0.00</td>
<td>0.06</td>
</tr>
<tr>
<td>111.</td>
<td>-</td>
<td>0.06</td>
<td>0.04</td>
<td>-</td>
<td>0.11</td>
<td></td>
</tr>
</tbody>
</table>

Unfortunately, time allowed for only two experiments. The results of these show that the frequency of DCV within a cohort remains at a low but fairly constant level.

The results of these experiments on mortality in the Ellis Beach and Araluen stocks can be summarized as follows. A temporal pattern in
mortality was identified in both stocks during the period August - November, 1985. This pattern suggested that the populations might have gone through severe bottle-necks at some point in time. DCV and DAV could be detected in these stocks in flies of between 27-30 days of age. The two viruses were detected either on their own, together, or not at all. It was found that there was no temporal pattern to the detection of the two viruses within cohorts, and neither was the mortality of cohorts characterised by the viruses present. It was not possible to explain any of the observed variation in mortality in the stocks by the effects of either DAV or DCV. Nevertheless, it was demonstrated that the removal of the viruses from the persistently infected stocks led to a decrease in mortality. The possibility exists that the decrease in mortality observed after virus-free stocks were established by dechorionation of the eggs from persistently infected mothers resulted as another consequence of the dechorination process. However, this alternative is not supported by the observation that reappearance of DAV within the HV stock was associated with a concomitant increase in the mortality of that stock (see Section 6.3.2.1D).

D. Relationship Between Mortality and Physical Parameters

The data presented in section 6.3.2.1B showed that in laboratory stocks persistently infected with DAV, there was no significant relationship between either adult density or body size (winglength) and mortality. Significant associations were found, however, between the frequency of DAV detection at times soon after eclosion (6-12 days) and mortality at later times (24 days). No features of the prevailing infection state within cohorts has been identified that can explain the variation in mortality observed in the Ellis Beach and Araluen stocks. Therefore, the question arose as to whether the
physical parameters of cohort density or mean body size (winglength) could explain any of the variation in mortality within these stocks?

Regressions of $M_{24}$ on adult density and female winglength (see, Section 6.3.2.1B) were calculated from the data both for the Ellis Beach and the Araluen stocks (Experimental periods 1B and 2 and period 1B respectively). The results are shown in Table 6.24

<table>
<thead>
<tr>
<th></th>
<th>EB</th>
<th>WL</th>
<th></th>
<th>AN</th>
<th>WL</th>
</tr>
</thead>
<tbody>
<tr>
<td>$b$</td>
<td>0.049</td>
<td>-0.012</td>
<td>0.115</td>
<td>-23.953</td>
<td></td>
</tr>
<tr>
<td>$n$</td>
<td>23</td>
<td>10</td>
<td>11</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>$r^2$</td>
<td>0.033</td>
<td>0.041</td>
<td>0.59</td>
<td>0.092</td>
<td></td>
</tr>
<tr>
<td>$p(a)$</td>
<td>0.707</td>
<td>0.345</td>
<td>12.946*</td>
<td>0.026</td>
<td></td>
</tr>
</tbody>
</table>

*= 0.05 > p > 0.025

(a) - degrees of freedom for $F = [1, n-2]$

The only significant regression found was that between density and $M_{24}$ in the Araluen laboratory stock, but the relationship between these two variables was not significant in the Ellis Beach data. Therefore, it would appear that a relationship between density and mortality is not a consistent feature of stocks persistently infected with DCV.

6.3.3.2 Egg Production

Egg production in cohorts from the persistently DCV infected stocks, Ellis Beach and Araluen, was measured as described in Section 6.3.2.3.

Figure 6.6 shows that the time-egg production curves (average egg production per female) in the DCV infected lines are as variable as
Figure 6.6 Daily egg production rates (D.E.P.) in stocks persistently infected with DCV

(Continued....)
Figure 6.6...continued

87. D.E.P.  
20

94. D.E.P.  
20

96. D.E.P.  

98. D.E.P.  

The analysis of variance of the egg production-time data from the Ellis Beach site revealed that there was a significant amount of the total variance accounted for by differences between the different periods. However, within both groups, a significantly greater proportion of the variance was explained by differences between different periods rather than by differences between sites.

The data for the analysis of variance of total egg production (based on density and wing length) and total egg production (based on density) are presented in Table 6.3.

The strong association between wing length (W) and density was not controlled for in this analysis. Therefore, this data will be summarized as wing length. An exception to this is that there was a significant relationship between wing length and density in the early stages of the experiment, but this was not controlled for in the analysis. Although in other cases there was a correlation between wing length and density, this was not significant.
those noted in the DAV infected lines (see Figure 6.4B), and that they are quite different from the standard egg production curve reported by other workers (see Figure 6.4A). These data on egg production rate were then used to calculate total egg-production between 4-20 days as described in Section 6.3.2.3. These data are presented in Table 6.25.

An analysis of variance of the egg production-time data from the Ellis Beach line revealed that time period could account for very little of the total variation, but that a significant amount of the total variation was accounted for by differences between cohorts (Table 6.26). Within the Araluen stock, a significant amount of the total variation was accounted for by both time-period and cohort differences. However, within both stocks, a significantly greater proportion of the variance was explained by differences between cohorts rather than by differences between time periods.

Using the total egg production data for the 4-20 day period, regressions were calculated on female mortality ($T_{50}$ and $M_{24}$), initial density and winglength. The results are shown in Table 6.27.

Significant regressions of total egg production on density ($D$) and winglength ($WL$) were found amongst the Araluen cohorts. A significant regression of total egg production on density was found within the Ellis Beach experiments. The strong association between winglength and total egg production in the Araluen line is unusual amongst the lines studied (either DAV-infected or DCV-infected). However, this may well be an aberration, as winglength in experiments involving this stock was found to be significantly correlated with density ($r_{[5]}=-0.784$, $0.05>p>0.02$). As density was not controlled accurately this result is difficult to explain, although in other stocks there is no correlation between winglength and density (Ellis Beach, $r_{[10]}=-0.114$, $p>0.5$; Huonville, $r_{[7]}=0.541$, $n=9$, $0.1>p>0.05$ and Coffs Harbour, $r_{[8]}=-0.447$, $0.1>p>0.05$).
Table 6.25 Egg production in two-daily periods between 4 and 20 days in persistently DCV infected stocks

<table>
<thead>
<tr>
<th>Expt No</th>
<th>Stock</th>
<th>2-4</th>
<th>4-6</th>
<th>6-8</th>
<th>8-10</th>
<th>10-12</th>
<th>12-14</th>
<th>14-18</th>
<th>18-20</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>75. AN</td>
<td></td>
<td>663</td>
<td>288</td>
<td>150</td>
<td>66</td>
<td>19</td>
<td>33</td>
<td>60</td>
<td>60</td>
<td>1339</td>
</tr>
<tr>
<td>76. AN</td>
<td></td>
<td>697</td>
<td>764</td>
<td>850</td>
<td>586</td>
<td>250</td>
<td>180</td>
<td>196</td>
<td>227</td>
<td>3750</td>
</tr>
<tr>
<td>77. AN</td>
<td></td>
<td>578</td>
<td>715</td>
<td>847</td>
<td>870</td>
<td>780</td>
<td>719</td>
<td>684</td>
<td>641</td>
<td>5834</td>
</tr>
<tr>
<td>84. AN</td>
<td></td>
<td>1232</td>
<td>2483</td>
<td>2898</td>
<td>2778</td>
<td>2510</td>
<td>2080</td>
<td>1870</td>
<td>1573</td>
<td>17424</td>
</tr>
<tr>
<td>87. AN</td>
<td></td>
<td>1100</td>
<td>1776</td>
<td>2493</td>
<td>2967</td>
<td>3049</td>
<td>2527</td>
<td>1740</td>
<td>1293</td>
<td>16945</td>
</tr>
<tr>
<td>94. EB</td>
<td></td>
<td>1044</td>
<td>841</td>
<td>444</td>
<td>226</td>
<td>89</td>
<td>116</td>
<td>154</td>
<td>148</td>
<td>3062</td>
</tr>
<tr>
<td>96. EB</td>
<td></td>
<td>190</td>
<td>100</td>
<td>729</td>
<td>1734</td>
<td>1944</td>
<td>1421</td>
<td>1195</td>
<td>1235</td>
<td>8548</td>
</tr>
<tr>
<td>98. EB</td>
<td></td>
<td>2259</td>
<td>2279</td>
<td>1886</td>
<td>1429</td>
<td>1121</td>
<td>1076</td>
<td>1110</td>
<td>1097</td>
<td>12257</td>
</tr>
<tr>
<td>100. EB</td>
<td></td>
<td>1962</td>
<td>2632</td>
<td>3274</td>
<td>3695</td>
<td>4077</td>
<td>3916</td>
<td>3332</td>
<td>2520</td>
<td>25408</td>
</tr>
<tr>
<td>104. EB</td>
<td></td>
<td>723</td>
<td>1061</td>
<td>1402</td>
<td>1746</td>
<td>1916</td>
<td>2174</td>
<td>2268</td>
<td>2214</td>
<td>13504</td>
</tr>
</tbody>
</table>
### Table 6.26 Analysis of variance of egg production in two-day periods in Ellis Beach and Araluen laboratory stocks

#### A. Ellis Beach

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS($10^{-7}$)</th>
<th>df</th>
<th>MS</th>
<th>L(a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>0.1501</td>
<td>7</td>
<td>0.0214</td>
<td>0.556</td>
</tr>
<tr>
<td>Experiment</td>
<td>3.4045</td>
<td>4</td>
<td>0.8511</td>
<td>22.106***</td>
</tr>
<tr>
<td>Residual</td>
<td>1.0782</td>
<td>28</td>
<td>0.0385</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4.6238</td>
<td>39</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### B. Araluen

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS($10^{-7}$)</th>
<th>df</th>
<th>MS</th>
<th>F(a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td>0.2553</td>
<td>7</td>
<td>0.0365</td>
<td>2.158*</td>
</tr>
<tr>
<td>Experiment</td>
<td>2.8794</td>
<td>4</td>
<td>0.7198</td>
<td>42.594***</td>
</tr>
<tr>
<td>Residual</td>
<td>0.47315</td>
<td>28</td>
<td>0.0169</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>3.6078</td>
<td>39</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(a) - degrees of freedom for F = [df days/experiment, df residual]

* = p<0.05  ** = p<0.01  *** = p<0.001
Table 6.27 Regressions of total egg production between 4-20 days on female mortality ($T_{50}$ and $M_{24}$), initial density (D) and winglength (WL) for data from Ellis Beach and Araluen laboratory stocks.

A. Ellis Beach

<table>
<thead>
<tr>
<th></th>
<th>$M_{24}$</th>
<th>$T_{50}$</th>
<th>D</th>
<th>WL</th>
</tr>
</thead>
<tbody>
<tr>
<td>b</td>
<td>-263.32</td>
<td>585.09</td>
<td>-291.74</td>
<td>6945.38</td>
</tr>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>$r^2$</td>
<td>0.460</td>
<td>0.536</td>
<td>0.929</td>
<td>0.122</td>
</tr>
<tr>
<td>f</td>
<td>2.525</td>
<td>3.467</td>
<td>39.361**</td>
<td>2.532</td>
</tr>
</tbody>
</table>

B. Araluen

<table>
<thead>
<tr>
<th></th>
<th>$M_{24}$ (a)</th>
<th>$T_{50}$</th>
<th>D</th>
<th>WL</th>
</tr>
</thead>
<tbody>
<tr>
<td>b</td>
<td>-324.68</td>
<td>681.85</td>
<td>-86.69</td>
<td>61476.96</td>
</tr>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>$r^2$</td>
<td>0.623</td>
<td>0.648</td>
<td>0.785</td>
<td>0.982</td>
</tr>
<tr>
<td>f</td>
<td>5.531</td>
<td>4.937</td>
<td>10.921*</td>
<td>103.764**</td>
</tr>
</tbody>
</table>

*= 0.05>p>0.01, **= 0.01>p>0.001

(a) - $M_{24}$ in experiment 74 was taken as 100%.
Although the regressions of egg production on either of the mortality parameters, \( T_{50} \) and \( M_{24} \) were not significant they do explain a large proportion of the variation in egg production. Nevertheless it would seem that, in stocks persistently infected with DCV, density has a more significant effect on total egg production within cohorts than any of the other variables tested.

6.4 DISCUSSION

6.4.1 Introduction

Very few studies to date have focussed on the effect that the picorna-like viruses of *Drosophila* have upon their host. In fact, few studies have been directed towards these areas for small RNA virus of insects. The persistently infected stocks established during the course of this study from natural populations offered an opportunity to identify and study the effects that DAV and DCV might have upon *D. melanogaster*. The results presented in Section 6.3.1 demonstrate that it was difficult to define what constitutes the major persistent infection within the *D. melanogaster* stocks studied. A variety of techniques had to be applied to differentiate qualitatively between infection states in the various stocks. The definition of a persistent DAV-infection used in this chapter was when a stock produced mostly DAV upon self-titration and only DAV was detectable *in situ*. A persistent DCV infection refers to stocks that produce mostly DCV upon self-titration and DCV is detectable *in situ* (along with DAV in most instances). The following discussion will concentrate first upon the results from the persistently DAV-infected lines, Huonville (HV), Tamar Valley (TR), Coffs Harbour (HD) and Cardwell (Mangoes) (CaM) and secondly on results from the persistently DCV infected lines, Ellis Beach (EB) and Araluen (AN). A discussion on points of general relevance will be presented at the end of the chapter.
6.4.2 Persistently DAV-Infected Stocks

Cumulative mortality at 24 days ($M_{24}$) and time at which 50% of a cohort was dead ($T_{50}$) were used as indexes of mortality in the present study. $M_{24}$ was found to be the most useful parameter in the persistently DAV-infected stocks because in most instances cumulative mortality did not reach 50% by 27-30 days when experiments were generally terminated. Considerable heterogeneity was found in $M_{24}$ amongst the four persistently DAV-infected stocks.

Previous studies have reported that there is a relationship between mortality and adult density (Pearl and Parker, 1922b; Pearl et al., 1927) and body size (Tantawy and El-Helw, 1966). No significant association could be found between either of these parameters and $M_{24}$ in any of the persistently DAV-infected stocks studied. Furthermore, DAV infection frequency at 27-30 days was found to always be in the range of 75-100% (Table 6.4) and could not account for the observed variation in $M_{24}$.

David and Plus (1971) demonstrated that there was a slight reduction in longevity associated with DPV infection transmitted by contact. To investigate this possibility i.e. the relationship between the presence of virus and increased mortality, virus-free lines were established from the Huonville and Coffs Harbour stocks (see Section 6.3.2.1D). Highly significant differences in mean $M_{24}$'s were found between the virus-free stocks and the parental, persistently infected stocks from which they were derived. In addition, the Huonville virus-free stock was discovered to have become reinfected with DAV. The reappearance of DAV in this stock was associated with an increase in its endogenous mortality. These observations suggest that an increase in $M_{24}$ was associated with the presence of DAV.

The high frequency at 27-30 days suggested that the frequency of DAV infection within a cohort increased through time. This was indeed
found to be the case. Moreover, significant associations were found between infection frequency at 6 and 12 days and $M_{24}$. However, no such association was found between $M_{24}$ and infection frequency at 18 days. Jousset and Plus (1975) showed that DAV could be transmitted by females to their progeny in the cytoplasm of the egg and from adult to adult by contact. Results presented in Chapter 5 (Section 5.3.7) showed that DAV could be transmitted from infected males to uninfected females and from infected females to uninfected males. In whichever direction the virus was transmitted there was a considerable delay (12-15 days) between the initial contact and detection of the virus in the recipient (uninfected) sex. This implies that the virus takes between 12-15 days to be transmitted and to replicate to a detectable level. Therefore, the frequency of infection detectable within the donor (infected) sex immediately prior to when the virus is first detected in the recipient sex appears to reflect flies that are infected at eclosion. As an association was found between infection frequencies at 6 and 12 days and $M_{24}$, the implication is that $M_{24}$ is related to the frequency of flies infected at eclosion. If this is the case then there is a delay between the virus reaching a detectable level in these flies and causing mortality.

From injection experiments the delay between detection of virus and any noticeable mortality effect was found to be around 4 days. To test for the magnitude of the delay between virus infection frequency and a noticeable mortality effect regressions of cumulative percentage mortality at 12, 15, 18, 21, 24, 27 and 30 days on infection frequency at 12 days were calculated for the eight persistently infected cohorts used in the previous analyses (see Section 6.3.2.2A). The results are presented in Table 6.28.
Table 6.28 Regressions of cumulative percentage mortality at day x on female infection frequency at day 12

<table>
<thead>
<tr>
<th>DAY</th>
<th>12</th>
<th>15</th>
<th>18</th>
<th>21</th>
<th>24</th>
<th>27</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>b</td>
<td>0.024</td>
<td>0.148</td>
<td>0.329</td>
<td>0.439</td>
<td>0.455</td>
<td>0.421</td>
<td>0.381</td>
</tr>
<tr>
<td>r^2</td>
<td>0.024</td>
<td>0.262</td>
<td>0.584</td>
<td>0.701</td>
<td>0.712</td>
<td>0.604</td>
<td>0.623</td>
</tr>
<tr>
<td>f</td>
<td>0.144</td>
<td>2.125</td>
<td>7.273*</td>
<td>14.068**</td>
<td>14.864**</td>
<td>9.145*</td>
<td>9.903*</td>
</tr>
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<tr>
<td>* = p&lt;0.05</td>
<td>** = p&lt;0.01</td>
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</table>

Significant regressions were found between female infection frequency at 12 days and cumulative percentage mortality at 18, 21, 24, 27 and 30 days. From these data it would appear that the delay between virus infection frequency at 12 days and a detectable mortality response is in the region of 6 days. Similar results were obtained from regressions of female infection frequency at 6 days and cumulative percentage mortality at the times shown in Table 6.28.

One model that can explain the association between M_{24} and infection frequency at 6 and 12 days, but not at 18 days, postulates that flies that acquire the virus before eclosion have a lower life-expectancy than those that acquire the virus later. Around 12 days is when virus begins to be detectable in flies that acquire DAV by contact. Hence included among those flies in which virus is detected at 12 days are flies that were infected at eclosion and some that have acquired the virus secondarily. However, cumulative mortality does not show much increase before 12 days (see Figure 6.7). Therefore at 12 days mortality due to the virus is low and although most of the flies that were infected at eclosion have detectable levels of the virus they have not yet died. At 18 days, however, a greater proportion of the flies that have detectable levels of the virus have acquired it secondarily. This proportion is also increased by the fact...
Figure 6.7 Mean cumulative percentage mortality (C.P.M.) curves for Huonville (HV), Coffs Harbour (HD) and Tamar Valley (TR) stocks.

HV: C.P.M. (%)

HD: C.P.M. (%)

TR: C.P.M. (%)

The C.P.M. is hypothesized to be related to the proportion of flies that emerge infected and the cumulative mortality curve will have distinct phases. The first phase would be sigmoidal, and plateau after all flies infected in a given batch have died. The second phase will be exponentially, terminating when all flies are dead. The sigmoidal mean cumulative percentage mortality curves for Huonville, Tamar Valley and Coffs Harbour stocks are shown in Figure 6.7. These curves do suggest that the models are appropriate to assess the behavior of the curve between 25-30 days. This suggests that there is variation in the response of individual flies and the rate of the infection and that each curve is possible that this variation is a reflection of the different effects of infection. Generally, this topic requires further work.
that flies that had been infected at eclosion have begun to die (see Figure 6.7). As $M_{24}$ is hypothesized to be related to the proportion of flies that emerged infected with DAV, the association between this parameter and infection frequency at 18 days disappears.

One prediction of this model, that follows if the life-expectancy of flies that emerge infected is sufficiently different from that of flies that acquire the virus secondarily, is that the cumulative percentage mortality curve will have two distinct phases. The first phase would be sigmoidal, and plateau after all flies infected at eclosion have died. The second phase will be exponential, terminating when all flies are dead. The mean cumulative percentage mortality curves for the Huonville, Tamar Valley and Coffs Harbour stocks are shown in Figure 6.7. These curves do appear to be sigmoidal and begin to plateau around 24-27 days. Unfortunately, experiments were terminated in this study at between 27-30 days, therefore, it is not possible to assess the behaviour of the curve beyond this time. Nevertheless, one interesting feature of the mortality curves is the gradual increase in C.P.M. until the apparent plateau at approximately 24-27 days. This suggests that there is variation in the response of individual flies to high levels of DAV infection and that death occurs between 18-27 for flies that are infected at eclosion. However, it is possible that this variation is a reflection of various levels of pre-eclosion/eclosion infection. Clearly this topic requires further work, particularly in relation to the mode of virus acquisition as few studies have investigated the effects that small RNA viruses have upon longevity. DAV (and also DPV) provide the opportunity to study such effects as it is known to be transmitted both in the cytoplasm of the egg and by contact.

It would appear from the evidence presented thus far, that any reduction in the life-span of flies infected at eclosion is limited to
the later stages in life i.e. >18 days post eclosion. Jousset and Plus (1975) demonstrated that transmission of DAV from mother to progeny via the cytoplasm of the egg ceases when mothers are greater than approximately 16 days of age. This age appears to correlate with the time at which individuals infected at eclosion are beginning to die from the effects of the viral infection (see Figure 6.7).

As the selective disadvantages of DAV infection, at least with respect to mortality, appear to be limited to the later stages of life, the question arose as to whether DAV was able to exert any selective pressure pre-imago. Other results have shown that direct contamination of diet did not effect the viability of larvae (see Section 5.3.6.1) or that larvae from persistently infected mothers were no less capable of developing through to imagos (see Section 5.3.6.2). However, these experiments had used larvae removed from their normal conditions of development i.e. relatively high larval density and a certain degree of contact with their parents. Initial observations had indicated that there was a slight deficiency in male progeny from persistently DAV-infected stocks. Further to this it had also been found that post-eclosion:

(i) male mortality was always less than female mortality
(ii) there was a consistently lower infection frequency in males relative to females

Possibly some mechanism exists such that males are being preferentially selected against before reaching the imago stage in persistently DAV-infected stocks.

Further investigation did reveal that there was a consistent deficiency of male progeny arising from persistently DAV-infected stocks. The deficiency also exhibited a characteristic association with the age of the mothers (see Figure 6.3). A significant regression was found between female infection frequency at 12 days and progeny sex-
ratios. As discussed above the infection frequency at 6 and 12 days appears to reflect the proportion of flies emerging that are infected with DAV. At 12 days the indications are that DAV has reached a high level (i.e. approximately $2 \times 10^{10}$ particles per fly) in most flies that were carrying the infection at eclosion, although any mortality due to the presence of the virus has not yet occurred (see Figure 6.7). As Jousset and Plus (1975) demonstrated, DAV is not transmitted to the progeny of females persistently infected with DAV after about 16 days post-eclosion. Therefore, from these data it would appear that the observed deficiency in male progeny in persistently-DAV infected stocks is related to the proportion of females infected at eclosion and the level of infection in these females. Exactly why, and by what mechanism, virus ceases to be transmitted directly to progeny after about 16 days is not known.

The phenomenon of skewed sex-ratios is well documented in *Drosophila* and characteristically appears as the production of virtually all female progeny. Unisexual progeny appear to be the result of preferential mortality of male zygotes (Malogolowkin et al., 1959). The agent responsible for causing the sex-ratio (SR) condition in *D. willistoni* and *D. nebulosa* is a spirochaete and can be transmitted by inoculation into other species e.g. *D. melanogaster* (Poulson and Sakaguchi, 1961). The SR condition of *D. bifasciata*, although having many of the characteristics of the SR condition of *D. willistoni* and *D. nebulosa* - production of unisexual progeny, maternally/cytoplasmically inherited - does not appear to be associated with a spirochaete or any other microscopic organism (Leventhal, 1966). Although not unequivocally established, the SR agent of *D. bifasciata* appears to be a virus (Leventhal, 1966). Although they are not the result of the same agent, the SR conditions of *D. willistoni/D. nebulosa* and that of *D. bifasciata* are of interest.
as they both selectively affect male zygotes through a maternally inherited factor. The deficiency of male progeny observed arising from persistently DAV-infected stocks, while not as severe as SR in *D. willistoni, D. nebulosa* and *D. bifasciata*, does have certain similarities. For instance, the DAV associated condition appears to be tied to maternal/cytoplasmic transmission. Further study of this phenomena and its association with DAV is obviously necessary to understand the mechanisms whereby DAV can be maintained within a population, and the effects that it might have in the population. It is also of interest to understand why such a diverse selection of pathogenic agents target male zygotes/larvae.

Another life-history parameter of *D. melanogaster* which DAV might affect is egg production. In most previous studies on egg production (e.g. McMillan et al., 1970; Hanson and Ferris, 1929; David et al., 1974) measurements were made on single females. However, the present study utilized a different experimental design in which egg production was measured at four-daily intervals between 4 and 20 days post-eclosion on groups of females. Such an experimental design was decided upon for several reasons, but primarily, because interactions between members of a cohort were considered to be important in terms of virus behaviour. Initial density was not closely controlled as it was not known what effects etherisation had upon DAV infected individuals. Later results showed that etherization has very little effect on mortality in persistently DAV-infected stocks. Recent studies have also demonstrated that repeated CO₂ anaesthesia does not affect either egg-production or life-expectancy in *D. melanogaster* (Partridge et al., 1986). Unfortunately it was not possible to repeat experiments controlling for initial density, but this should be borne in mind as one parameter that needs to be controlled more closely in future experiments.
From previous studies in which data were obtained from single females (see Figure 6.4A), the general pattern of egg production rate was found to be a rapid increase to a peak at between 4-6 days. This was followed by an exponential decay until the egg production rate reaches zero (usually between 40-50 days). The shape of this curve suggested that the period between 4 and 20 days would be a suitable time over which to measure egg production, because it is post peak-production and includes the period of greatest change in the egg-production rate.

The results showed that the egg-production rate curves were very variable within the persistently DAV-infected cohorts (see Figure 6.4B). Furthermore, these curves bore little resemblance to the "standard" egg-production rate curve reported by other workers (see Figure 6.4A). Using the values of egg-production rate and the associated mortality data it was possible to calculate egg production in two-daily periods for a nominal population of 100 females (see Section 6.3.2.3). There was more variation between cohorts in this data than there was between the two-day periods for both the Huonville and Coffs Harbour stocks (Table 6.18).

A highly significant regression was found between $M_{24}$ and the total egg-production between 4 and 20 days (the sum of two-daily egg production values). However, female mortality was accounted for in the calculation of egg production in each two day period, and hence in total egg production. Therefore, it might be reasonable to suppose that there would be a strong relationship between total egg production and mortality that occurs as a result of the method used to calculate total egg production. To test whether this was indeed the case, the mortality curves for the HV experiments were imposed on the "standard" egg production curve (see Figure 5.8A), and total egg production calculated for each hypothetical cohort. A simple regression of these
total egg production data on $M_{24}$ was then calculated. The regression was not significant ($F_{[1,6]}=5.585$, $0.10>p>0.05$). This procedure was then repeated, only this time the mortality curves were superimposed on the mean egg-production curve for the Huonville data. A simple regression of total egg production on $M_{24}$ was then calculated. The regression was significant ($F_{[1,6]}=7.18$, $0.04>p>0.025$, $r^2=0.545$).

However, the relationship between total egg production in these data is not as strong as in the raw data set, and the regression explains only 54.5% of the variation. Therefore, it would seem that total egg production is closely linked to the time-mortality curve from which it is calculated (see Section 6.3.2.3).

Pearl (1932) showed that increased density was associated with a reversible decrease in egg production rate and that, as soon as the density was decreased there was a concomitant increase in egg production rate. Hence, increasing density of females appears to bring about a decrease in egg production rate by inducing females to function at sub-maximal rates. This suggests that females, to a certain extent, interfere with the egg-laying of their contemporaries. However, if density had an effect in such a way as this in the experiments reported here, then it would be predicted that the egg production rate should rise steadily over the course of time i.e. as cumulative percentage mortality increases. Figure 6.4B shows that this is not the case, which suggests that a much more complex interaction occurs between density, mortality and egg production than is discernable from the data presented in this study.
6.4.3 Persistently DCV-Infected Stocks

As discussed at the beginning of this section, four qualitatively different infection states involving DAV and DCV could be identified in laboratory stocks of *D. melanogaster* (see Table 6.23). Two stocks were found to produce mostly DCV upon self-titration while DCV and DAV were detectable by screening individual flies from these stocks. These two stocks, Ellis Beach and Araluen, are considered to have DCV as their major persistent infection.

Mortality within these two stocks was found to be associated with the presence of their persistent virus infections, as stocks established from surface sterilized eggs have significantly reduced $M_{24}$ (see Section 6.3.3.1B). Within these two lines four different infection states could be identified in individual cohorts on the basis of the viruses detectable in flies surviving at 27-30 days: DCV only present, DAV only present, DCV and DAV present and no viruses present. During the period in which experiments were carried out there was no evidence for a temporal sequence in the distribution of these four infection states. In addition, there was no difference between the mean mortalities ($M_{24}$) of the cohorts within each of the four groups.

During the experimental period August-November, 1985, the Ellis Beach and the Araluen stocks were observed to go through a phase where endogenous mortality within the stocks was very high. Plus et al. (1975b) observed a similar situation in stocks infected with the highly pathogenic DCV strain DCV$_0$, although in Plus et al.’s stocks the effect appears to have been much more prolonged and was reversible by establishing lines from surface sterilized eggs. Therefore, what probably occurred in the Ellis Beach and Araluen stocks was that the endogenous DCV in these stocks became much more pathogenic. During this phase of higher pathogenicity either, flies more resistant to DCV
were selected for, or a less severe strain of DCV was selected. The timing of this phenomenon is interesting as it happened in both stocks concurrently. It is unlikely that the flies became infected at this time because as long as 6 months prior to the observed increase in mortality, DCV was readily isolatable from both stocks by self-titration. Therefore, it would appear that the effect was precipitated by some other factor. Unfortunately the nature of this factor cannot be surmised from the data available.

Unlike the situation described for DAV, very little variation in DCV infection frequency was found within cohorts from stocks infected with DCV (see Table 6.23). The physical parameters of density (D) and winglength (WL) were analysed for their relationship with $M_{24}$ in the Ellis Beach and Araluen stocks. The only significant regression was between density and mortality in the Araluen stock. The relationship between density and mortality was not a consistent feature of DCV infected stocks, and the positive relationship found in the Araluen data would not have been predicted from the results of Pearl and Parker (1922).

Considerable variation was observed in egg production rate curves in the Araluen and Ellis Beach stocks (Figure 6.6). As with the DAV infected lines, most of the variation in egg production was between cohorts within the two persistently DCV-infected stocks (Table 6.26). However, in contrast to the DAV infected stocks, density was found to give the most significant regression on total egg production between 4-20 days (Table 6.27). In the persistently DAV-infected Huonville stock mortality was the only parameter that had a significant association with total egg production between 4-20 days. The association of egg production with density in the two DCV infected lines, but not in the DAV infected line, is not due to the heterogeneity in the densities tested in the experiments (Bartlett's
test for homogeneity of variances, $B_c = 2.76$, $0.25 > p > 0.1$). The association of egg production with different variables in stocks that are predominantly infected with DAV or DCV may well be a real qualitative difference in the effects of the two viruses upon egg production, although this subject undoubtedly requires more study.

6.4.4 Summary

From the results presented in this chapter it would appear that one important qualitative difference between DAV and DCV is in the mode of their pathogenic action. DAV increases in frequency within a cohort through time, due to the additive effects of primary (hereditary/cytoplasmic) transmission and secondary transmission by contact. However, a delay of at least 4 days is observed between the virus reaching high titres (in excess of $6 \times 10^7$ particles per fly) and beginning to cause mortality. At these high titres the total amount of virus present in individual flies is in the region of $2.5 \mu$g, or approximately 0.25% of the flies' total weight (approximately 1mg). In terms of nucleic acid, the viral RNA would represents in the region of 4% of the flies' total nucleic acid ($21 \mu$g per fly - estimated spectrophotometrically). This is a considerable proportion of the flies' nucleic acid resources devoted to viral RNA. The main site of DAV replication is in the intestine and Malpighian tubules (Brun and Plus, 1980). Other insect viruses that primarily infect the gut of their host e.g. cytoplasmic polyhedrosis viruses, can be tolerated at high titres without producing death. Thus, it would appear that the pathogenic effect of DAV is the result of the virus reaching high titres in certain tissues, where its continued presence over a period of time disrupts the hosts normal physiological functions to such an extent that death eventually results.
In contrast to DAV, DCV was detected at very low frequencies within cohorts. However, within these cohorts, DCV could invariably be detected in cadavers collected from approximately 3 days onwards. This suggests that DCV has a different growth-mortality pattern from DAV inasmuch as it replicates to detectable levels (approximately $2 \times 10^7$ particles/fly - see Chapter 4) whereupon it causes death almost immediately. This is supported by the cytopathology of the virus, as it has been observed to multiply freely in the tracheal cells, especially those around the cerebral ganglion (see Brun and Plus, 1980). Virus infection of nervous tissue in insects has been found to be associated with rapid death in a number of insects (e.g. acute bee paralysis virus, Bailey and Gibbs, 1964; CrPV, Reinganum et al., 1970). The rapid pathogenic effect of DCV once it reaches detectable levels is most probably the underlying reason behind the observed low levels of DCV infection frequency within cohorts i.e. as soon as the virus reaches a detectable level the infected individual dies.

In conclusion, mortality at 24 days within persistently-DAV infected cohorts is related to infection frequency at earlier times in the cohorts history, which is itself a reflection of the frequency of individuals in the cohort that are infected prior to eclosion. These results indicate that DAV has a differential pathogenic effect depending on the mode of its transmission i.e. cytoplasmically inherited from infected mothers or secondarily acquired by contact. DAV acquired as a cytoplasmically inherited infection appears to be associated with preferential pre-imago mortality of males. It is also possible that DAV affects egg-production indirectly, as a strong relationship is found between total egg-production between 4-20 days and mortality at 24 days. The mechanism by which this may occur is yet to be identified.
Within DCV-infected stocks any effects due to the presence of the virus are much more difficult to elucidate because of the low levels of variability in DCV infection frequency. However, the presence of DCV is associated with an increase in endogenous mortality within persistently infected stocks, and this mortality can be quite dramatic at times, causing mortality of 50% of a cohort within 4-5 days.
CHAPTER 7 THE DISTRIBUTION OF DAV AND DCV IN NATURAL POPULATIONS OF DROSOPHILA

7.1 INTRODUCTION

In the introductory chapter of this thesis I outlined four features of the Australian Drosophila fauna that make them an interesting group in which to study their viruses. In summary these are:

(i) a number of species of Drosophila may be found together at any one collecting site where they form a community of closely related species. Many of these species are considered to be cosmopolitan (Patterson and Stone, 1952), and occur in communities along with endemic species of Drosophila. These communities form points of contact between native and introduced species within the genus and pose questions about whether viruses of introduced species have become adapted to the endemic species and vice versa.

(ii) along the eastern seaboard of Australia there is a continuous distribution of Drosophila communities, without obvious isolating barriers, in ecologically diverse habitats. Although the composition of these communities varies with latitude, D. melanogaster and D. simulans are found as components of nearly all communities that are sampled.

In addition, D. melanogaster and D. simulans have been shown to be genetically differentiated along a latitudinal transect for certain enzyme loci and for common chromosome inversions (see Section 1.4.3). Latitudinal variation has also been described in physiological parameters such as cold resistance (Parsons, 1977), and desiccation resistance (Parsons, 1980) in Australian D. melanogaster and D. simulans populations. There is also evidence in other parts of the
world for latitudinal variation in body weight and ovariole number (David and Boquet, 1975) and in total egg production (Bouletreau-Merle et al., 1982). These features of the geographical differentiation of Drosophila species offer the possibility to test whether the ecological and/or genetic heterogeneity is reflected in the geographical distribution of their viruses.

Most of the work so far reported in this study has been concerned with the characterisation of DCV and DAV, and the effects that these viruses have, under laboratory conditions, on D. melanogaster. The work described in this chapter focusses on the distribution of the viruses in natural populations of D. melanogaster and within the communities in which this species occurs.

7.2 MATERIALS AND METHODS

7.2.1 Collection of Drosophila

Between November 1983 and December 1986, 34 collections of Drosophila were made from 25 sites within Australia (see Table 7.1). These collections were made mainly at sites along the eastern seaboard and span 26.7° of latitude from Daintree (16.3°S) in the north to Cygnet (43°S) in the south. Collections were also made on an E-W transect as far west as the Barossa Valley (139°E). Eight sites were collected twice and one site three times; collections at these sites were spaced at least 9 months apart.

All collections were made by sweep-netting over suitable substrates. Flies were snap frozen in liquid nitrogen upon collection and shipped to Canberra where they were kept frozen at -20°C until used.
Table 7.1 Details of localities at which Drosophila collections were made

Sites 1-19 form a north-south transect down the eastern seaboard of the continent. Sites 20-25 (below dotted line), form a west-east transect across the continent.

(1) Abbreviation of collection site name

(2) Major substrate type at collection locality

REF = human refuse
MAN = fallen mangoes
PAW = fallen paw-paws
BAN = banana dumps at commercial plantations
FUJ = fallen fujoa fruit
PEA = peach dump at commercial orchard
WP = grape pressing at wineries, mostly skins and stalks
APP = apple waste after juice-pressing
WW = washings from fermentation vats at winery.
Table 7.1 Details of localities at which *Drosophila* collections were made

<table>
<thead>
<tr>
<th>Locality (Qld)</th>
<th>Abbr.</th>
<th>Lat(S)</th>
<th>Long(E)</th>
<th>Date</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Daintree (Qld)</td>
<td>DN</td>
<td>16°15'</td>
<td>145°19'</td>
<td>11/12/85</td>
<td>REF</td>
</tr>
<tr>
<td>2. Port Douglas (Qld)</td>
<td>PD</td>
<td>16°32'</td>
<td>145°19'</td>
<td>20/11/83</td>
<td>MAN</td>
</tr>
<tr>
<td>3. Ellis Beach (Qld)</td>
<td>EB</td>
<td>16°44'</td>
<td>145°39'</td>
<td>19/11/83</td>
<td>MAN</td>
</tr>
<tr>
<td>4. Cairns (Qld)</td>
<td>CN</td>
<td>16°55'</td>
<td>145°46'</td>
<td>19/11/83</td>
<td>MAN</td>
</tr>
<tr>
<td>5. Mareeba (Qld)</td>
<td>MA</td>
<td>17°00'</td>
<td>145°26'</td>
<td>21/11/83</td>
<td>PAW</td>
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<tr>
<td>6. Ravenshoe (Qld)</td>
<td>RV</td>
<td>17°36'</td>
<td>145°29'</td>
<td>01/12/85</td>
<td>REF</td>
</tr>
<tr>
<td>7. Mission Beach (Qld)</td>
<td>MB</td>
<td>17°56'</td>
<td>146°06'</td>
<td>01/12/85</td>
<td>MAN</td>
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<tr>
<td>8. Cardwell (North) (Qld)</td>
<td>CaB</td>
<td>18°15'</td>
<td>146°01'</td>
<td>01/12/85</td>
<td>BAN</td>
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<tr>
<td>9. Cardwell (South) (Qld)</td>
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<td></td>
<td>22/11/83</td>
<td>MAN</td>
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<td>10. Gympie (Qld)</td>
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<td>26°11'</td>
<td>152°53'</td>
<td>11/12/85</td>
<td>BAN</td>
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<tr>
<td>11. Kin Kin (Qld)</td>
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<td>26°16'</td>
<td>152°40'</td>
<td>24/03/85</td>
<td>FUJ</td>
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<td>153°09'</td>
<td>17/07/84</td>
<td>BAN</td>
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<td>13. Canberra (ACT)</td>
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<td>35°17'</td>
<td>149°13'</td>
<td>14/04/85</td>
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<td>14. Araluen (NSW)</td>
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<td>35°39'</td>
<td>149°49'</td>
<td>01/02/84</td>
<td>PEA</td>
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<td>15. Lakes Entrance (Vic)</td>
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<td>37°53'</td>
<td>148°00'</td>
<td>19/02/86</td>
<td>REF</td>
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<td>146°55'</td>
<td>03/05/84</td>
<td>WP</td>
</tr>
<tr>
<td>17. Hobart (Tas)</td>
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<td>42°53'</td>
<td>147°19'</td>
<td>30/04/84</td>
<td>WP</td>
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<td>HV</td>
<td>43°02'</td>
<td>147°04'</td>
<td>01/05/84</td>
<td>APP</td>
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<td>19. Cygnet (Tas)</td>
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<td>43°09'</td>
<td>147°05'</td>
<td>01/05/84</td>
<td>APP</td>
</tr>
<tr>
<td>20. Griffith (NSW)</td>
<td>GF</td>
<td>34°15'</td>
<td>146°11'</td>
<td>11/05/84</td>
<td>WP</td>
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<td>21. Nagambie (Vic)</td>
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<td>145°10'</td>
<td>14/05/86</td>
<td>WP</td>
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<td>37°17'</td>
<td>142°56'</td>
<td>15/05/86</td>
<td>WP</td>
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<td>23. Mildura (Vic)</td>
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<td>142°10'</td>
<td>14/05/84</td>
<td>WP</td>
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<td>139°03'</td>
<td>12/05/86</td>
<td>WP</td>
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<td>25. Barossa Valley</td>
<td>BV_H</td>
<td>34°32'</td>
<td>138°58'</td>
<td>12/05/86</td>
<td>WP</td>
</tr>
</tbody>
</table>
7.2.2 Sorting of Flies

Flies were sorted using the criteria of Bock (1976). *D. melanogaster* and *D. simulans* females were separated by the black posterior marking on the sixth abdominal tergite (Gallo, 1973). This character is about 95% reliable in Australian populations of *D. melanogaster* and *D. simulans* (W.D. Atkinson, pers. comm.). Due to the frozen state in which flies were kept, there was considerable dehydration of specimens and a concomitant collapse of their abdomens. For this reason, it was not possible to separate males of *D. melanogaster* from those of *D. simulans* in collections where both species were present. In these samples the relative frequency of *D. melanogaster* and *D. simulans* was calculated from the relative proportions of females.

Many collections north of Cardwell (18.3°S) were found to contain *D. ananassae* and *D. pseudoananassae*. The females of these species (and the males which have a similar colouration to the females and lack the black posterior to the abdomen characteristic of *D. melanogaster/simulans* males) could generally be separated from *D. melanogaster/simulans* by their less conspicuous abdominal tergite markings (Bock, 1976). Individuals for which there was any degree of uncertainty were discarded. Males of *D. ananassae/pseudoananassae* can be separated by reference to the sex-comb. However, no attempt was made to separate males of these two species in the present study as much of the material was damaged after freezing. Furthermore, it is not possible to separate the females of these two species. Therefore, all *D. ananassae* and *D. pseudoananassae* have been classified together as *D. ananassae*.

In all collections containing males with entirely black posteriors to the abdomen, reference was made to the sex-comb of at least fifty flies to check for the presence of other species e.g. *D. pseudotakahashii*.
160

7.2.3 Dot-Blotting and Detection of DAV and DCV

Individual flies were ground in 200μl of distilled water containing 0.01% DEPC, and vacuum dot-blotted onto Hybond-N. Replicate filters were made for each group of samples and probed both for DAV and DCV as described in Chapter 4 (see Figure 4.6).

7.3 RESULTS

7.3.1 Species Compositions of Drosophila Communities

The species compositions of Drosophila collections made in the course of this thesis are presented in Table 7.2.

The general pattern of the composition of these communities is very much in agreement with what has previously been found with urban Drosophila communities in Australia (Figure 7.1)

Due to the wide variety of substrates and the different seasons (particularly in southern regions) in which collections were made I feel that a rigorous treatment of these data is not warranted. Nevertheless, it is possible to make some qualitative statements about the trends in the species compositions of these collections. Two major groups of species assemblages are commonly encountered;

(i) northern type assemblages: characterised by the presence of D. ananassae and D. sulfurigaster in relatively high proportion. This type of assemblage extends as far south as Cardwell. Neither of these two species were encountered in collections south of this point. It should be noted that there is a large sampling gap in this study between Cardwell and Gympie (the next most southerly collection) which spans approximately 6° of latitude. D. ananassae and D. sulfurigaster reach the southern limit of their distribution in this area, neither species occurring south
Table 7.2 Species compositions of *Drosophila* collections made in this study

The species in each collection are represented as a total proportion of the flies collected. - indicates that the species was not found at all in the collection. + indicates that the species was present but represented less than 0.01 as a proportion of the total number of flies.

(a) Species abbreviations:

- **mel** - *Drosophila (Sophophora) melanogaster*
- **sim** - *D. (Sophophora) simulans*
- **mel/sim** - *D. melanogaster/ simulans* males that could not be separated in frozen samples (see Section 7.2.2)
- **ana** - *D. (Sophophora) ananassae/ pseudoananassae* - these two species were inseparable in frozen collections (see Section 7.2.2)
- **sul** - *D. (Drosophila) sulfurigaster*
- **hyd** - *D. (Drosophila) hydei*
- **imm** - *D. (Drosophila) immigrans*
- **bus** - *D. (Dorsilopha) busckii*
- **bry** - *D. (Scaptodrosophila) bryani*
- **lat** - *D. (Scaptodrosophila) lattivitata*
Table 7.2 Species compositions of *Drosophila* communities sampled in this study

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Figure 7.1 Composition of Drosophila communities commonly encountered in Australian collections

D. melanogaster
D. simulans
D. ananassae
D. pseudoananassae
D. sulfurigaster

D. melanogaster
D. simulans
D. immigrans
D. hydei
of 21°S (Anderson, 1985). The typical species composition of northern-type assemblages is given below with the average proportion of each species in parentheses; D. melanogaster (0.31), D. simulans (0.13), D. ananassae (0.30) and D. sulfurigaster (0.16). Minor components of these northern communities are D. bryani (relatively common where it is found), D. hydei and D. busckii. Although sometimes recorded from rainforests in Queensland (Bock, 1976), D. immigrans was not encountered in any collections north of Gympie (26.2°S).

(ii) the southern-type assemblage: characterised by the presence of D. hydei and D. immigrans together, and relatively high proportions of D. melanogaster/simulans. Generally, in these communities D. melanogaster and D. simulans do not occur together at high frequencies.

On the basis of these two species assemblages and their geographical distributions, I would suggest that they reflect two major ecotypic zones for Drosophila communities. The limit of the northern zone appears to be reflected in the distribution of D. ananassae and D. sulfurigaster.

7.3.2 The Distribution of DAV and DCV in Australian Drosophila Communities

7.3.2.1 Detection of Infected Populations

The results of the survey for DAV in Australian Drosophila communities are shown in Table 7.3, and those for DCV in Table 7.4. The incidence of infected Drosophila communities is summarized in Figure 7.2.
Table 7.3 Frequency of DAV detection in Australian Drosophila communities. The number of positive individuals found is expressed over the total number of flies screened.

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</table>

(a) Species abbreviations are shown in the legend to Figure 7.2.
Table 7.4 Frequency of DCV detection in Australian Drosophila communities. The number of positive individuals found is expressed over the total number of flies screened.

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<th>SPECIES(a)</th>
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<th>mel/sim</th>
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<th>imm</th>
<th>bus</th>
<th>bry</th>
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<tbody>
<tr>
<td>TOTAL</td>
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<td>524</td>
<td>536</td>
<td>315</td>
<td>342</td>
<td>454</td>
<td>262</td>
<td>4</td>
<td>145</td>
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</table>

(a) Species abbreviations are shown in the legend to Figure 7.2
Figure 7.2 Distribution of communities found to be infected with DAV and DCV.

Site numbers are as shown in Table 7.1.

〇 = No virus detected

● = DAV only detected

■ = DCV only detected

★ = DAV and DCV detected in the same community
The cDNA hybridization technique utilized in the present study was developed because it gives data on the frequency of infected flies within a population. However, the question arises as to whether the technique provides a reasonable indication of whether or not DAV or DCV are present in a population.

Of the 34 Drosophila communities screened for the presence of DAV and DCV, 8 (24%) were found to be infected with DAV and 6 (18%) with DCV. Overall, 12 (35%) out of the 34 Drosophila communities screened contained at least one virus.

Plus et al. (1975b) tested 29 D. melanogaster populations from around the world, 9 of these (31%) were found to be infected with DAV and/or DCV. Their study utilized the successive passages technique which relies on the passaging of sterile isolates from a sample of flies, three or four times through virus-free D. melanogaster before testing for the presence of viruses serologically and/or by electron microscopy. This technique should be capable of detecting very small quantities of virus in the initial sample as the titre of the virus is increased by each passage. Therefore, successive passaging of isolates from natural populations of Drosophila should be an accurate indication of whether or not a virus is present at all in a population.

There is good agreement between the present study and that of Plus et al. in the incidence of infected communities detected. This suggests that cDNA hybridization as used in the present study provides a good indication of whether a virus is present or not in a community. However, it is more likely that this "good agreement" is merely fortuitous.

In the following sections, all comparisons of infection frequencies between and within populations and communities are based only on the data from those populations in which the viruses have been detected.
7.3.2.2 The Distribution of DAV and DCV Within Drosophila Communities

Inspection of the data presented in Tables 7.3 and 7.4, reveals two features of the virus distributions with respect to species:

(i) DAV was detected most frequently in *D. melanogaster* and *D. simulans*, but it was also detected in *D. immigrans* and *D. sulfurigaster*,

(ii) DCV was detected only in *D. melanogaster* and *D. simulans* and not in any of the other species screened.

Due to the difficulty in separating *D. melanogaster* and *D. simulans* males prior to screening (see Section 7.2.2), a direct comparison cannot be made between the frequency of DAV infection in these two species. The distribution of DAV between males and females is shown in Table 7.5. A test of homogeneity between the frequency of infection in females of these two species reveals no significant species difference ($\chi^2_1 = 0.86$, $p > 0.5$). In communities where *D. melanogaster* males and females could be readily separated i.e. *D. simulans* not present, there was no difference in infection frequency between the two sexes ($\chi^2_1 = 1.53$, $0.5 > p > 0.2$). When the data for both species are pooled and males and females are compared, there is found to be no significant heterogeneity between sexes ($\chi^2_1 = 1.42$, $0.5 > p > 0.0.2$). Therefore, it would appear that in natural populations, DAV infection frequency is homogeneous in *D. simulans* and *D. melanogaster*. In addition, males are as frequently infected as females. As no differences could be found in the infection frequency between *D. melanogaster* and *D. simulans*, data for the two species were pooled for further analyses. No significant differences could be found between the infection frequency in *D. melanogaster* and *D. simulans* females ($\chi^2_1 = 0.208$, $p > 0.5$) or between males and females, when the data for both species were pooled ($\chi^2_1 = 0.241$, $p > 0.5$)
Table 7.5 Frequency of DAV detection in males and females of *D. melanogaster* and *D. simulans*.

The number of infected individuals in each class are expressed over the total number of individuals from that class that were screened. The species abbreviations are as shown in Figure 7.2. Sample numbers and abbreviations are as shown in Table 7.1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>mel female</th>
<th>mel male</th>
<th>sim female</th>
<th>sim male</th>
<th>mel/sim male</th>
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<td>-</td>
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<td>-</td>
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<td>9. CaM (1985)</td>
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<td>2/18</td>
<td>-</td>
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<tr>
<td>10. GY (1985)</td>
<td>1/47</td>
<td>-</td>
<td>0/60</td>
<td>-</td>
<td>2/48</td>
</tr>
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<td>12. HD (1984)</td>
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<td>1/18</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>1/36</td>
<td>-</td>
<td>4/76</td>
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<td><strong>149</strong></td>
<td><strong>137</strong></td>
<td><strong>0</strong></td>
<td><strong>238</strong></td>
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</table>

**Total no. screened of each class**

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<tr>
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<th>CaM</th>
<th>GY</th>
<th>HD</th>
<th>GF</th>
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<td>644</td>
<td>375</td>
<td>149</td>
<td>512</td>
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</table>
The frequency of DAV infection was compared between *D. melanogaster/simulans* and the other commonly occurring species (*D. ananassae, D. sulfurigaster, D. hydei* and *D. immigrans*). Comparisons were only made using data from infected populations, and for each pairwise comparison only those populations were used in which the two species occurred together. The results of these comparisons are shown in Table 7.6.

DAV infection frequencies are homogenous between *D. melanogaster/simulans* and *D. ananassae, D. sulfurigaster* and *D. immigrans*. However, the number of infected populations for which data for these comparisons was available is small. Nonetheless, the difference between the DAV infection frequency in *D. melanogaster/simulans* and *D. hydei* is highly significant. Although DAV will grow after injection in both *D. melanogaster/simulans* and *D. hydei*, the heterogeneity observed in natural populations strongly suggests that under these conditions there are different host-virus interactions in operation.

**Table 7.6** $\chi^2$ values obtained in comparisons between the infection frequency of DAV in *D. melanogaster/simulans* and other *Drosophila* species with which they were associated in natural populations

<table>
<thead>
<tr>
<th></th>
<th>ananassae</th>
<th>sulfurigaster</th>
<th>hydei</th>
<th>immigrans</th>
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<tbody>
<tr>
<td><em>melanogaster/simulans</em></td>
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<td></td>
<td>1.69</td>
<td>0.39</td>
<td>11.41***</td>
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</table>

*** = $p<0.001$
7.3.2.3 Geographical Distribution of DAV and DCV in *D. melanogaster/simulans* Populations

In their study on the geographical distribution of picornaviruses (DAV, DCV and DPV) in natural populations of *D. melanogaster* from around the world, Plus et al. (1975b) found a marked bias in the incidence of infected populations. A greater proportion of infected populations was found in warmer than in more temperate climates.

On the basis of the species composition of *Drosophila* communities in Australia (see Figure 7.1), two major ecotypic zones for *Drosophila* can be proposed; a northern, tropical zone and a southern more temperate zone. Given the results of Plus et al. (1975b), the distribution of infected populations would not be expected to be homogeneous across these two zones.

When the incidences of DAV infected populations within the northern and southern ecotypic zones are compared, no significant heterogeneity in virus distribution is found ($\chi^2_1 = 0.65$, $0.5 > p > 0.2$).

However, a further subdivision of the data is suggested by the geographical distribution of the populations sampled. There are large gaps in this distribution between Cardwell and the next most northerly collection (Gympie), and between Coffs Harbour and the next most northerly collection (Canberra). The northern zone previously described is limited by the southernmost distribution of *D. ananassae* and *D. sulfurigaster*. Nevertheless, it is possible that a latitudinally varying climatic zone overlies the two zones previously described. To test this, the winglength of flies from each of the three putative zones were measured. Winglength is a good reflection of developmental temperature (Tantawy and Mallah, 1961). The results of these measurements are presented in Figure 7.3 (the available temperature data for the sites at which collections were made during this study are given in Appendix 1). Significant associations were
Figure 7.3 Female winglength of flies collected from natural populations (measured in graticule units) plotted against $T_{\text{Min}}$.

The graph shows the relationship between wing length and $T_{\text{Min}}$ for female flies. The data points are plotted against the temperature range from 10 to 22°C. The graph indicates a negative correlation between wing length and temperature, with wing length decreasing as temperature increases.

The following points are observed:
- Points for 'HD' are marked with an 'o' symbol.
- The points are spread across the temperature range, with a trend line indicating the decrease in wing length with increasing temperature.

The text accompanying the figure provides additional context and analysis, including statistical comparisons and conclusions drawn from the data presented in the graph.

The text also mentions that significant heterogeneity was found amongst the incidences of DAV-infected populations, and a comparison was made of the incidence of DAV-infected populations in the northern and intermediate zones (between Adelaide and Canberra). No significant deviation from homogeneity was detected amongst the remaining populations (1954 and 1955), suggesting that the incidences of DAV in these populations did not differ significantly.
found both between $T_{\text{Max}}$ (average daily maximum temperature in the month before, and the month of collection) and $T_{\text{Min}}$ (average daily minimum temperature in the month before, and the month of collection). This demonstrates that the populations between Cardwell and Araluen do form a third zone - at least on the basis of developmental temperature at the time of collection.

A comparison was made of the incidence of infected populations within these three zones. Significant heterogeneity was found amongst the incidences of DAV-infected populations ($\chi^2 = 26.65, p<0.001$). Comparisons between the northern and intermediate (between Araluen and Cardwell), and between the southern and intermediate zone, each revealed significant heterogeneity ($\chi^2_1 = 4.421, 0.05>p>0.02$ and $\chi^2_1 = 9.769, 0.01>p>0.001$ respectively). Close inspection of the frequencies within infected populations reveals that the two Coffs Harbour populations (1984 and 1985) have a much higher frequency than any of the other populations (see Table 7.7).

If the frequencies of DAV infection within infected populations are compared, there is significant deviation from homogeneity (tested using the G-test of Sokal and Rohlf (1969); $G=18.16, \chi^2_{0.05[7]}=14.07, 0.05>p>0.02$). When the data for the Coffs Harbour populations (HD) are removed, no significant deviation from homogeneity is detected amongst the remaining populations ($G=1.10, \chi^2_{0.05[5]}=11.07, p>0.5$). Thus, the heterogeneity is primarily due to the high frequencies of DAV found at Coffs Harbour in 1984 and 1985.

DCV was detected in 6 out of the 34 populations screened. The frequencies within these populations are given in Table 7.7. No significant heterogeneity was found between these populations ($G=2.12, \chi^2_{0.05[5]}=11.07, p>0.5$). A comparison of the homogeneity of the incidences of DCV infected populations between the 3 zones revealed no significant differences ($\chi^2_2=3.705, 0.5>p>0.2$)
Table 7.7 Infection frequencies of DAV and DCV within *D. melanogaster/simulans* populations. Sample numbers and abbreviations are as shown in Table 7.1.

### A. DAV infected populations

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<th>Uninfected</th>
<th>Frequency(%)</th>
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<td>155</td>
<td>1.9</td>
</tr>
<tr>
<td>(1985)</td>
<td>13</td>
<td>204</td>
<td>6.8</td>
</tr>
<tr>
<td>20. GF (1984)</td>
<td>1</td>
<td>168</td>
<td>0.6</td>
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**TOTALS**

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<th>Uninfected</th>
<th>Frequency(%)</th>
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<td>1046</td>
<td>2.4</td>
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### B. DCV infected populations

<table>
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<th>Frequency(%)</th>
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<td>1.0</td>
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<tr>
<td>5. MA (1985)</td>
<td>1</td>
<td>107</td>
<td>0.9</td>
</tr>
<tr>
<td>9. CaM (1983)</td>
<td>1</td>
<td>89</td>
<td>1.1</td>
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<tr>
<td>10. GY (1985)</td>
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<td>16. TR (1984)</td>
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</tr>
<tr>
<td>20. GF (1984)</td>
<td>1</td>
<td>167</td>
<td>0.6</td>
</tr>
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**TOTALS**

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<tr>
<th></th>
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<th>Uninfected</th>
<th>Frequency(%)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>7</td>
<td>762</td>
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7.4 DISCUSSION

The discovery of DAV and DCV in laboratory stocks of
D. melanogaster established from natural populations collected within
Australia, indicated that these two viruses were likely to be found in
natural populations of D. melanogaster (see Section 3.4). This is
indeed the case both for DAV and DCV. Furthermore, the frequency of
infection within populations was found to be homogenous in
D. melanogaster and D. simulans for both viruses.

One of the questions posed in the introduction to this chapter
was: which species is/are the major host(s) for DAV and DCV in natural
populations/communities of Drosophila? From the data presented in
Section 5.3.6 and the study of Jousset (1976), it would appear that
the highly pathogenic effect of DCV, after injection, is limited to
D. melanogaster and D. simulans. DCV was found not to replicate to
detectable levels in any species other than D. melanogaster and
D. simulans. Therefore, the failure to detect DCV in any species other
than D. melanogaster/simulans in the natural communities that were
screened is not surprising. The range of species in which DCV has a
pathogenic effect, and in which the virus is able to replicate after
injection, suggests that D. melanogaster and D. simulans are the major
hosts for DCV in natural populations.

In contrast to DCV, DAV was found to replicate readily, after
injection, in D. immigrans, D. hydei, D. sulfurigaster and
D. ananassae (see Section 5.3.6). DAV was detected in D. immigrans and
D. sulfurigaster from natural populations but not in D. hydei or
D. ananassae. The only significant difference in infection frequencies
between any pair of species was for D. melanogaster/simulans and
D. hydei. This difference suggests that D. hydei is not a major host
for DAV in natural populations. As DAV replicates readily in D. hydei
after injection, its absence from this species in natural populations
could be explained in three ways:
(i) inability of the virus to be transmitted from the major host in natural populations to *D. hydei* and to reach tissues where it is able to replicate e.g. a gut barrier to the virus transmission,

(ii) inability of the virus to replicate to the levels detectable in the dot-blot assay i.e. *D. hydei* is only a semi-permissive host, or

(iii) micro-habitat separation between *D. hydei* and the major host species so that *D. hydei* never comes into contact with DAV in natural environments.

Although micro-habitat separation and the spread of viruses between species in different micro-habitats has been little studied, the DAV-*Drosophila* system appears to be worthwhile to investigate. For instance, it is known that there is some degree of separation between *D. melanogaster/simulans* and *D. hydei* in habitat preference (Atkinson, 1977). Furthermore, as the results in Chapter 5 show, DAV is readily detectable in *D. melanogaster*, and has a distinctive pattern of increasing frequency within a given cohort.

In summary, *D. melanogaster/simulans* appear to be the major host for DCV in natural communities of *Drosophila*. However, it is not possible to ascertain which *Drosophila* species is the major host for DAV from the data currently available.

Another feature of interest in the data described in this chapter is the detection of DAV in the Ellis Beach (1983) population of *D. sulfurigaster*. From the widespread geographic distribution of DAV and DCV (see Plus et al., 1975b) it is most probable that these two viruses were originally viruses of one of the cosmopolitan species: *D. melanogaster* *D. simulans*, *D. immigrans* or *D. hydei*. *D. sulfurigaster* is an endemic species to the Australasian region and to S.E. Asia. Therefore, if DAV did indeed arrive in Australia along with the
cosmopolitan species of Drosophila, it has become adapted to growth in
D. sulfurigaster under natural conditions. More extensive sampling of
D. sulfurigaster populations will provide the opportunity to isolate
and characterise the DAV present in this species much more fully, and
will enable studies on the changes associated with widening of host
range in insect viruses to be carried out. It will be possible to
assess whether changes in the ecology or in the biochemistry of the
virus are associated with a widening of the host range.

The frequency of infection in D. melanogaster/simulans populations
was generally in the range of 0.5-2.5%, the only exception to this
being the frequency of DAV at Coffs Harbour (HD) in 1984 and 1985. Few
data were hitherto available on the frequency of infection of
Drosophila viruses in natural populations. Indeed there is very little
data on small RNA viruses in any natural insect populations. Fleuriet
(1976), in the only report of the frequency of infection of any
Drosophila virus in natural populations, stated that the frequency of
sigma virus infection was invariably in the range 10-20%.

Wigley and Scotti (1983) showed that the frequency of CrPV in
populations of the New Zealand small field cricket, Pterinemobius
nigrovus, varied over the course of the summer months between
approximately 10% and 40%. Another of the insect picornaviruses,
Gonometa virus, was found to have an infection frequency of
approximately 75% in field populations of Gonometa podocarpi (Harrap
et al., 1966). The latter frequency appears unusually high, but the
virus was found to be associated with a noticeable disease state, and
was isolated as a response to the disease epizootic. Another small RNA
virus, the Nodavirus of the black beetle Heteronychus arator, was
found at frequencies of 25% in third instar larvae and 7% in adults
from field populations (Longworth and Archibald, 1975).
The general picture that emerges from these data is that the frequency of infection of small RNA viruses in field populations of their hosts is in excess of 10%. This frequency is considerably higher than the levels detected in most of the *D. melanogaster/simulans* populations sampled in this study. Why then should the frequency of DAV and DCV be lower than that found for other small RNA viruses? From the data available at present it is not possible to answer this question. Nonetheless, it is clear that considerable herogeneity in the frequency of virus infection occurred between DAV-infected populations, with the frequency at Coffs Harbour (HD) in 1984 and 1985 being much higher than at any other site. The frequencies observed in these two collections i.e. 12.9% and 6.8%, are in the range of the frequencies found for sigma virus and other small RNA viruses. Therefore, an understanding of why the virus is at much higher frequency in these samples, relative to those from other sites, may help to explain why the frequency of DAV and DCV is generally so low.

Two basic mechanisms can be postulated for maintaining a higher than normal frequency of virus within a population:

(i) increased resistance of the host inhibiting the virus multiplying to levels at which it has deleterious effects, or

(ii) environmental/ecological conditions that facilitate the virus reaching relatively high frequencies

From the data presented in Chapter 5, there is no evidence to support the theory that the Coffs Harbour (HD) population was any more resistant to DAV than other populations studied. In terms of reduced longevity associated with the persistent DAV infection, Coffs Harbour appears to be intermediate between Huonville (high mortality) and Cardwell (Mangoes) (low mortality). So, it would appear that different ecological/environmental conditions might be responsible for the high infection frequency at Coffs Harbour. Two, not necessarily exclusive,
alternatives can be offered as to how environment might affect virus infection frequency:

(i) growth/ transmission conditions are better suited to the virus frequency increasing within the population

(ii) the environment exerts less selective pressure on infected individuals.

No evidence is available to either support or discount either of these possibilities. However, several environmental/ecological factors can be postulated that could affect virus frequency within natural populations of D. melanogaster:

(i) temperature: most life-history and reproductive functions of D. melanogaster are at an optimum in the temperature range 18-22°C (e.g. Tantawy and Mallah, 1961; David and Clavel, 1966; McKenzie, 1975). If infected flies are preferentially selected against, then selection would probably be greater in more marginal environments.

(ii) seasonality: extreme seasonality where flies cannot breed in the winter months forces populations through narrow bottlenecks with the result that founder populations in the following spring are relatively small (e.g. McKenzie, 1975). Changes in population size, particularly with reference to seasonal changes, have been found to be associated with changes in genetic factors e.g frequency of certain enzyme alleles and chromosome inversions (Knibb, 1986). Furthermore, Fleuriet (1981a) demonstrated that sigma virus infection frequency in the first progeny of spring produced by flies that had overwintered as adults, was lower than in the last progeny of the previous autumn.

(iii) resource availability: if resources are plentiful at one site all of the year around, then successive generations are
present at that site. With little need for migration to find new resources, any virus-infected individuals would be present at the same site continuously.

(iv) population size: in large populations utilising the same resource, contact transmission of virus should be enhanced.

(v) larval competition: both inter- and intraspecific competition have been identified as affecting body size in natural *Drosophila* communities (Atkinson, 1979b). In environments where larval competition is high one might expect that selection against infected individuals would be greater than in environments where there is little competition.

On the basis of these factors we can make predictions about the types of environments in which we could expect to find certain levels of infection frequency. For instance, stable environments with little seasonality would be expected to support populations with relatively high frequencies, as would populations with plentiful resources capable of supporting a large population.

The environmental conditions at Coffs Harbour make it an interesting site for several reasons. The climatic conditions at this site are relatively stable, with warm summers and cool, but not cold, winters. Seasonality is not as well marked, in terms of temperature, as it is at sites further south. In addition, the temperature never falls consistently below $15^\circ C$ in the winter months, which probably enables *D. melanogaster* and other *Drosophila* species to breed all of the year round. In fact, both collections at this site were made during June/July, and although *D. melanogaster* was at a low frequency relative to *D. hydei* and *D. immigrans*, the number of flies was still fairly high. In the summer months *D. melanogaster* forms a much greater proportion of the population and the population at this site is very large (J. Gibson, pers comm). The site at Coffs Harbour where both
collections were made is in a banana and avocado plantation. At this particular site resources are available throughout the year within a relatively small area. At other sites where collections were made for this study however, the resources are much more ephemeral. For instance, at Cardwell (Mangoes) resources are only available in the mango fruiting season, which is relatively short and further decreased by the rapid decay of the fruits due to the high temperatures at this season. Therefore the resource is only available for a short time, and flies will then have to disperse to find new sites. At the more southerly sites e.g. Tamar Valley resources and suitable breeding temperatures in the area are only available for a very short period (3-5 weeks) i.e. vintage time at the winery, and in a small area. Overall, Coffs Harbour appears to be a site where conditions are suitable for breeding and resources are available all the year round, thus providing a relatively stable environment for D. melanogaster.

The Coffs Harbour site is clearly of the type that one would predict would support D. melanogaster populations with high DAV infection frequencies. The Coffs Harbour samples also lie within a group of collections in which the incidence of infected populations were particularly high. Unfortunately, only four collections were made between Cardwell and Araluen which comprise this "zone". Many questions therefore remain to be answered with regard to the putative zone: how far north and south does it extend and what environmental factors does it correlate with? How distinct is Coffs Harbour within this zone? i.e. is the high frequency observed there just an outlying population? Whatever the reasons are for the high frequency of DAV infection at Coffs Harbour, and the high incidence of infected populations in the "intermediate zone", it is notable that the geographic distribution of DAV does not appear to follow a latitudinal cline as described for certain genetic (see Section 1.4.3) and physiological (see Section 7.1) traits.
Finally, it should be remembered that whatever ecological factors control the distribution of DAV in natural populations, they cannot explain the data for DCV. As no geographical variation could be identified in the distribution of DCV, factors other than ecological must be invoked to explain its distribution.
The insect viruses can be divided into two major groups, the occluded and non-occluded viruses (see Section 1.2). The occluded viruses have their virions encapsulated in a large pseudocrystalline matrix termed the polyhedron or occlusion body. The size of this occlusion body (in the range of 0.5-15µm), coupled with the fact that most of the occluded viruses produce a recognisable disease state in their host, has undoubtedly aided in the isolation and identification of many of these viruses. In contrast, most of the non-occluded insect viruses identified to date do not cause any apparent symptoms in their host, while their small size does not aid either their discovery or identification. For these reasons, it is only by the use of modern procedures developed in the last 15-20 years that it has become feasible to study the small, non-occluded insect viruses. The potential for these studies has been further increased in the last ten years because of the advances made in the technology of virus detection and identification.

In the introduction to this thesis I outlined four major aims to which the work was directed:

(i) to isolate and characterise the viruses present in natural Australian populations of *D. melanogaster*,

(ii) to develop a sensitive and specific assay for the detection of the viruses found in these populations,

(iii) to ascertain the distribution of the viruses between and within natural communities of *Drosophila* in Australia,

(iv) to investigate the biology of the virus(es) isolated under laboratory conditions and to attempt to elucidate the possible effects that they might have in natural populations.
Discussions of the results of experiments directed to these aims have been presented at the end of the relevant data chapters. The intention of the following discussion is to integrate the results of the studies reported in this thesis and to relate them to other studies on non-occluded insect viruses. The initial part of this discussion will be concerned with the first two of the four aims listed on the previous page. As these primarily involve technical problems, the discussion will first focus on the difficulties associated with the study of insect viruses, and particularly the small non-occluded viruses of insects.

Most previous studies on the viruses of *Drosophila* have relied upon the passaging of isolates, suspected of containing virus, in a known virus-free strain of *D. melanogaster*. A major problem encountered early in the course of the present study was the maintenance of virus-free stocks of *D. melanogaster*. Although relatively easy to establish (see Sections 5.3.2 and 5.3.3), virus-free stocks of *D. melanogaster* did prove difficult to maintain. For instance, a virus-free line established from the persistently DAV-infected Huonville stock became reinfected with DAV within three months of its establishment. Considerable care was taken in the culturing of virus-free lines to minimise the risk of contamination. For instance, diet was made first thing in the morning before I had any contact with other *Drosophila* stocks or *Drosophila* homogenates, and all transfers and collections were carried out at this time. In addition, putative virus-free stocks were isolated from other stocks of *Drosophila* and maintained in an area where no other *Drosophila* work was in operation. Despite these precautions, virus-free stocks became reinfected. Unfortunately many of the facilities other workers employ in the maintenance of virus-free *Drosophila* stocks (A. Fleuriet, pers. comm.) were not available in the present study. These facilities include:
(i) a separate building in which virus-free lines can be maintained away from other Drosophila stocks, with stringent precautions to preclude stray flies from the environment,
(ii) facilities for the preparation of diet and culturing of the flies within the separate building
(iii) personnel with specific duties involving only the culturing of virus-free lines.

Communications with Dr. A. Fleuriet have however confirmed that, even under the stringent culturing conditions outlined above, virus-free stocks still become infected from time to time.

To circumvent the problem of not having a reliable stock of virus-free flies, the method of self-titration was developed (see Section 3.2.2). Although the method proved capable of providing material for the isolation of DAV and DCV from persistently infected stocks, it does have certain limitations:

(i) only viruses available as persistent infections in *D. melanogaster* stocks could be propagated and isolated,
(ii) mixed virus preparations were invariably produced from the stocks utilized in the present study.

Mixed virus populations are not a problem restricted to Drosophila and their associated viruses, and many reports of small isometric viruses in laboratory populations of insects reports commonly involve more than one type of virus. For instance, Reed and Desjardins (1978) observed small isometric virus-like particles of three different sizes (18nm, 30nm and 33nm in diameter) in the citrus red mite, *Pananychus citri*. Juckes (1970) identified 5 different virus-like particles (termed α, β, γ, δ and ε) in the in the pine emperor moth, *Nudaurelia cytherea capensis*. Two of these viruses, *Nudaurelia* β and ε viruses, although serologically distinct (Juckes et al., 1973) are morphologically and biophysically very similar (Juckes, 1979), and
the E virus was always found as a minor component of β virus preparations (Juckes, 1979). An even more extreme example of multiple virus infections is found in Australian populations of the honeybee (Anderson, 1986). In this study up to 4 small RNA viruses were found to be present in pupae from a single hive, and in some instances individual pupae were deduced to contain more than a single virus.

Mixed virus preparations present problems when developing an assay system, and it is important to be able to separate the viruses present in the mixed virus preparations. Probably the two most sensitive virus detection systems developed so far are enzyme linked immunosorbant assay (ELISA) and nucleic acid hybridization. Both techniques have their advantages and disadvantages and both are constrained to a certain extent by mixed virus preparations.

The limiting factor in using the ELISA technique is the specificity of the antisera used to immobilize and quantify the virus. Obviously problems are encountered if the antisera is specific for more than one virus e.g. the anti-DAV<sub>HV</sub> and anti-DAV<sub>HD</sub> sera produced from self-titrated DAV preparations (see Table 3.1) had specificity for DAV and DCV. This problem is not intractable and, for example, a monoclonal antibody could be produced against the virus of interest, although a method must be available to identify the virus against which the monoclonal antibody has been raised. Furthermore, monoclonal antibodies specific against a particular strain of a virus need not necessarily react with all closely related strains e.g. monoclonal antibodies raised against a type 3 poliovirus isolate have been found not to react with all type 3 isolates (Minor et al., 1982). Thus, in any study where a range of virus strains could be encountered, more than a single monoclonal antibody would have to be used to detect all strains of the virus present.
Results presented in the current study demonstrate that the development of a nucleic acid hybridization assay can also be hampered by mixed virus preparations (see Section 4.3.1.2). Fortunately, DAV and DCV can be separated electrophoretically (see Figures 3.2C and 4.4C), and this property was useful for identifying the virus from which the cloned cDNAs were derived.

The hybridization assays developed for DAV and DCV were capable of detecting the viruses in isolates from single D. melanogaster. Using these assays I was therefore able to address the third and fourth aims specified above, which are concerned with the biology and ecology of DAV and DCV, and it is to these topics that the following discussion will be addressed. Particular reference will be made to the results presented in Chapters 5, 6 and 7 and to assess to what extent they relate to the overall ecological strategy of DAV and DCV in natural populations of Drosophila. A broader appraisal of the biological strategies of the insect viruses as a whole, and the small non-occluded viruses in particular, will also be presented.

Viruses are, by definition, obligate intracellular parasites and in order to reproduce themselves they must continually find new replicative sites. As a consequence of their replication it is invariably the case that some cost is incurred by the host. Throughout the animal and plant kingdoms, viruses have evolved a vast array of strategies for finding new hosts e.g. transmission by direct contact between infected and uninfected host, hereditary transmission and the use of vectors. However, as parasites, viruses must achieve a balance between the way in which they are transmitted and the cost that they incur on their host.

The division of the insect viruses into the occluded and non-occluded viruses, based primarily on the structural difference produced by the presence or absence of the occlusion body, also
reflects a major ecological difference between the two groups in the ways in which they are transmitted, and hence, upon the effect that they exert on their host.

The occlusion body confers a relatively large degree of stability upon the occluded viruses. In the occluded form, infectious virus can remain in the environment for many months, either on the insect-host food plant (e.g. Entwistle and Adams, 1977; David and Gardiner, 1966) or in the soil (e.g. Jaques, 1970; Young and Yearian, 1979). In fact, the polyhedron confers such stability upon the virus, that infectious virus can be recovered from the faeces of birds (Entwistle et al., 1977) and mammals (Lautenschlager and Podgwaite, 1977). Most of the occluded viruses are usually associated with the death of their host, and in many cases cause widespread epizootics (e.g. Balch and Bird, 1944; Tanada, 1961; Entwistle, 1977). After the virus reaches high titres, and the death of the host ensues, the virus is dispersed into the environment via the infected cadavers. The stability of the virus thus ensures that it persists in the environment until it is able to find a new host.

DAV and DCV are both small non-occluded viruses (see Section 1.2), and their reproductive strategies differ from those of the occluded viruses. Preliminary studies with DAV and DCV (data not previously presented) have shown that they do not remain detectable by dot-blot hybridization assay in cadavers of flies for more than 48 hours. This suggests that both of these viruses are fairly labile, and DCV inocula stored overnight at 4°C loses about 40% of its pathogenicity (personal observation). Therefore, one might expect that DAV and DCV will differ from the occluded viruses in their modes of transmission and in the effects that they have upon their host. If, as a consequence of their relatively labile nature, small non-occluded viruses of insects do not disperse into the environment, then it may be inferred that they
remain closely associated with the host. Under such circumstances, a
c constraint placed upon the virus is that it cannot be too pathogenic,
as death of the host terminates its ability to be transmitted to new
hosts.

The studies reported in Chapter 6 demonstrated that as persistent
infections in laboratory cultures of *D. melanogaster*, DAV and DCV had
relatively low pathogenicity. Although the presence of DAV and DCV
within a stock increases the endogenous mortality of adult flies, the
increased mortality was not apparent until about 18 days post-eclosion
(See Section 6.3.2.1D and 6.3.3.1B). Data on DAV and DCV certainly
appear to support the prediction made above that small non-occluded
viruses will generally be non-pathogenic, and in laboratory stocks the
two viruses appear to function as chronic, rather than acute,
infections of *D. melanogaster*. So, the question arises: how are
chronic infections maintained and the virus transmitted within natural
populations?

In laboratory stocks of *D. melanogaster* persistently infected with
DAV, the virus appears to be transmitted in two ways:

(i) from adult to progeny through the egg,

(ii) from adult to adult by contact.

The first of these two modes of transmission leads to the
production of progeny that are infected at eclosion. After eclosion,
the virus replicates in the imagos to high levels (approximately
$2 \times 10^{10}$ particles/fly) in approximately 12 days (Section 5.3.7), during
which time the virus is also transmitted by contact to the uninfected
members of the cohort. At the high levels that the virus reaches, a
considerable proportion of the fly’s resources are devoted to the
virus (Section 6.4.4). Nonetheless, despite this apparent burden, the
flies are able to survive for at least 6 days after the virus reaches
a high titre i.e. before there is any noticeable increase in mortality
within the cohort. Indeed, many flies do not succumb until at least 12-15 days after the virus reaches a high titre. Hence, it would appear that although the virus does have a pathogenic effect upon *D. melanogaster* the effect is not manifest earlier than 18 days after eclosion.

Interestingly, Jousset and Plus (1975) demonstrated that females greater than 16 days of age no longer transmit DAV to their progeny in the cytoplasm of the egg. Therefore, it would seem that the virus does not exert any pathogenic effect until after it has ceased to be transmitted in a persistent manner via the cytoplasm of the egg. Such a strategy ensures that the virus is transmitted to as many progeny as is possible, thus providing the virus with the maximum number of new replicative sites.

Undoubtedly, transmission via the egg is a very stable way for a virus to be transmitted, and assuming that there is no preferential selection against individuals that are infected in this way, it ensures a constant transmission of the virus through successive generations. Many studies on small non-occluded viruses have reported some form of transovarial transmission e.g Reinganum et al., 1970; D’Arcy et al., 1981b and Comendador et al., 1986. So, it would appear that transmission of small non-occluded viruses of insects via the egg is the rule rather than the exception. As this mode of transmission is relatively stable and can ensure that the virus is passed onto new hosts in a persistent fashion, the question arose: what role do other modes of transmission play in natural populations?

* D. melanogaster* larvae can readily be infected *per os* with DPV and DCV, and by contact with adult cadavers rich in DCV (Jousset and Plus, 1975). DAV is readily transmitted by contact between adults and reaches high levels after approximately 12 days of contact (Section 5.3.7). Indeed, laboratory studies have shown that all of the
Drosophila viruses (with the exception of sigma) can be transmitted by contact or per os (see Table 1.4 and Brun and Plus, 1980 for a review). However, conditions in laboratory cultures are very different from those in natural populations. High density and confinement to a relatively small space, with no opportunity for dispersal, undoubtedly increase the efficiency of contact transmission in laboratory stocks. The question therefore arises; how might contact transmission be increased in natural populations of Drosophila?

High density will obviously help to increase the incidence of contact transmission, particularly when the same group of individuals are in contact for long periods of time. However, as the small occluded viruses do not appear to be very stable in the environment, other factors can be postulated that would increase the incidence of contact transmission. Specialised behaviour patterns could well increase the incidence of contact transmission within populations e.g. necrophagy or coprophagy, by reducing the effective time that the virus must remain in the environment.

Drosophila larvae can regularly be seen feeding on the soft parts of decaying adult cadavers in laboratory cultures. Although this may be the result of a general feeding pattern in which everything in the top 2-3mm of the diet is consumed, I have observed that on poor diet (agar and sucrose only) larvae will preferentially feed on, and around, decaying cadavers. Necrophagy is not an uncommon behavioural trait in insects, and Reinganum (1975) has observed that crickets will readily consume the remains of Antheraea eucalypti larvae infected with CrPV. Of course, one requirement of this mode of transmission is that infected individuals die in an environment where their remains can be consumed. Certainly, in the case of worker honeybees infected with sacbrood virus this does not occur, and the behaviour of the infected bees is modified to reduce the risk of contaminating other individuals.
in the colony. For instance, it has been observed that infected workers stop gathering pollen and feeding larvae before they disappear completely from the hive. So, although the virus infection does not increase the mortality of adult workers, it may be inferred that the infected workers do not die but just remove themselves from the hive (Bailey and Fernando, 1972).

The elucidation of virus transmission routes via specialised behaviour of the host requires detailed observations on the host and its behavioural patterns. With the study of the biology and ecology of small RNA viruses of insects still very much in its infancy, observations on the "fine-tuning" of virus-transmission are scant. This is an area that requires more research in the future if the mechanisms whereby small RNA viruses are maintained in their insect-host populations are to be elucidated.

One postulated consequence of transovariole transmission as a means of maintaining a virus within a population was that individuals infected in this way should not be preferentially selected against. However, among progeny arising from persistently DAV-infected stocks, there was a DAV-associated deficiency in males (see Section 6.3.2.2.B). Although there was no direct relationship between the observed deficiency in male progeny arising from the persistently DAV-infected stocks and larval density, density is almost certainly involved to some extent in increasing the selective pressure on infected larvae e.g. via reduced resource availability. However, selection against pre-adult males logically does not affect the viruses capability of being transmitted to new hosts, as there is no evidence to suggest that D. melanogaster males play any part in vertical transmission of DAV. Therefore, under sub-optimal conditions i.e. where not all larvae will survive, it appears that the virus selects less strongly against those individuals (females) that are capable of transmitting the virus to the next generation.
The available evidence suggests that overall, DAV has relatively low pathogenicity and the pathogenicity it exhibits in *D. melanogaster* is restricted to periods (greater than 18 days post-eclosion) and individuals (males), that do not affect its ability to be transmitted in a persistent manner. Therefore, it seems reasonable to suppose that the virus should occur at relatively high frequencies in natural populations. The laboratory studies indicated that the frequency of individuals infected at eclosion is generally in the region of 50% (see the frequencies in Table 6.13 for 12 days post-eclosion). This value represents the proportion of individuals that are infected in a continuous/persistent manner between generations and might be expected to be the frequency found in natural populations. However, the frequencies observed in Australian populations of *D. melanogaster* were mostly in the range of 1-2% (Section 7.3.2.2). These frequencies are considerably lower than those reported for sigma virus (Fleuriet, 1976), or for several small non-occluded viruses in natural populations (e.g. Longworth and Archibald, 1975; Wigley and Scotti, 1983). Despite these generally low frequencies of DAV infection in the populations sampled, significant heterogeneity was found between the infection frequencies in the populations (Section 7.3.2.2) that were infected. Two collections made at Coffs Harbour in 1984 and 1985 were found to have infection frequencies of 12% and 9% which contributed markedly to the observed heterogeneity in infection frequencies. So, what could explain this heterogeneity?

Preliminary evidence suggests that a possible explanation for the high frequencies recorded at this site in consecutive years resides in the relative ecological stability that the site provides for *D. melanogaster*. *Drosophila* are opportunistic breeders and utilize widely separated, discrete resources e.g. the fallen fruit under a tree. Furthermore, as primarily "microbial grazers" i.e. feeding on the
micro-flora and fauna of decaying fruits, their breeding habitats are only available for a few generations while the fruit is in a suitable state of decay. Under these ephemeral conditions it could be argued that transmission between generations in association with the egg would be more important than contact transmission. Nevertheless certain circumstances might increase contact transmission in natural populations.

One feature of the Coffs Harbour collection site which distinguishes it from all of the other collection sites is that resources are available throughout the year in a relatively small area. Couple this with the fact that the climate is suitable for D. melanogaster to breed all of the year round, and it would appear that this site probably supports a continuous population of overlapping cohorts throughout the year. In such an environment, it might be postulated that contact transmission of DAV would have a larger role in determining infection frequencies than at other more ephemeral sites. Therefore, the possibility exists that the observed high frequency of DAV at Coffs Harbour is due to the additive effects of maternal and contact transmission. It follows then that, in most populations, the level of DAV infection is very much depressed, as the environment does not provide the stability necessary to ensure a constant "flow" of virus through the population. It should also be remembered that although this ecological explanation fits the data for the Coffs Harbour populations, the infection frequencies in general are still much lower than would be predicted from the laboratory studies. Moreover, this is not a situation confined to the D. melanogaster/DAV system, and the frequencies of sigma virus in cage populations (Fleuriet, 1981a, 1982) is generally found to be lower than in natural populations (Fleuriet, 1976, 1986). Thus, it would appear that there are more complex systems in operation in natural
populations that maintain the frequency of infection at relatively low levels. Nonetheless, the *D. melanogaster* /DAV system is undoubtedly a useful one to study to gain further understanding of how *Drosophila* viruses and, in the broader context, small non-occluded insect viruses behave in natural populations, exposed to different environmental conditions.

Traditionally DAV and DCV have always been treated as belonging to two different biological groups (see Brun and Plus, 1980). However, the indications are that, at least in terms of their pathogenicity, these two viruses have similar effects in laboratory populations (e.g. see Section 5.3.2). Unfortunately, studies on laboratory populations of *D. melanogaster* persistently infected with DCV proved more difficult than studies on DAV stocks. The greatest confounding factor was that DAV was invariably found along with DCV as a dual infection. Although DCV was also shown, by self-titration, to be present in the persistently DAV-infected stocks, its expression appeared to be suppressed in these stocks, and hence DCV had very little effect relative to that of DAV. Therefore, within stocks carrying DCV as a major persistent infection, it is difficult to apportion the observed effects on life-history parameters to the two viruses. Nevertheless, certain qualitative differences were found between the two major infection types (i.e. DAV- and DCV-infection - see Section 6.3.1):

(i) both DCV-infected stocks were observed to go through a period of high mortality (see Section 6.3.3.1A) not observed in the DAV infected stocks

(ii) egg-production was not associated with mortality in the DCV-infected stocks (Section 6.3.3.3) whereas mortality was strongly associated with mortality in the DAV-infected stocks (Section 6.3.2.3).
The qualitative differences observed between persistently DAV-infected and DCV-infected stocks in the present study, together with data from other studies on DCV (Plus et al., 1975b; Jousset and Plus, 1975), suggest that there may be considerable differences in the mechanisms by which DAV and DCV are distributed and maintained in natural populations.

One difference between DAV and DCV, is that DCV appears to have a much shorter delay between reaching high levels within a fly and causing mortality (see Section 6.4.4). Despite this relatively rapid pathogenicity, DCV appears to be tolerated as a persistent infection. However, within the cultures that were studied (Ellis Beach and Araluen), it is possible that at some point in their laboratory history the flies were selected for resistance to DCV, or that a less pathogenic strain of DCV arose and was selected for (see Section 6.4.3). In experiments in which a local isolate of DCV along with a highly pathogenic isolate of DCV from overseas (DCV₀ - see Plus et al., 1978) were injected into strains of D. melanogaster derived from different Australian localities, no difference was found in the relative pathogenicity of the two isolates. This evidence suggests that the Australian strains of D. melanogaster have some form of resistance factor against DCV (see Sections 5.3.3 and 5.3.4).

The presence of resistance factors against DCV has previously been reported for stocks of D. melanogaster "from different geographic origins" (Plus and Goubovsky, 1980), although no systematic survey of their distribution has been undertaken. Obviously the presence of resistance factors within natural populations will play a very large role in determining the demographic behaviour of a virus in natural populations. For instance, it is known that there is a geographical correlation between one of the resistance factors against sigma virus and the form of the virus that is sensitive to it (Fleuriet, 1986).
Such a system has evolved despite the fact that sigma virus has not been found to have any detrimental effects on *D. melanogaster* in laboratory populations.

An understanding of the distribution of these putative resistance factors in natural populations is obviously important if we are to understand how DCV is maintained in Australian populations of *D. melanogaster*. For instance, it is important to find out whether there is any geographical correlation in the distribution of the virus and the "resistance factors", and how they inhibit DCV.

In toto, my results have shown that it was possible to develop an assay system for the two viruses commonly found in laboratory populations of *D. melanogaster* - DAV and DCV. The cDNA assay system developed for each virus was specific and could be used to reliably detect the viruses in isolates from single flies from laboratory and natural populations. The assay technique was used in laboratory studies to demonstrate that DAV and DCV both reduce the life-expectancy of *D. melanogaster*, and that DAV preferentially selects against pre-adult (embryos/larvae) males. The mechanisms by which these two viruses are transmitted between individuals in a population appear to fit into a general scheme in which the viruses behave as persistent, chronic infections of the host. Nevertheless, the mechanisms by which DAV could be shown to be transmitted in laboratory populations does not appear to act as efficiently in most natural populations, as the frequency of DAV infection is lower than might be expected. Geographically related heterogeneity in the frequency of infection in the natural populations sampled implies that genetic, ecological and environmental factors play an important role in limiting the spread of DAV in natural populations.


Entwistle, P.F. (1977). The development of an epizootic of a nuclear polyhedrosis virus disease in European spruce sawfly, Gilpinia hercyniae. International Colloquium on Invertebrate


Appendix 1  Temperature data for Drosophila collection sites. Access no. = file number of the nearest weather station to the collection site. Data obtained from the Bureau of Meterology, Canberra.

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(a) = mean data from 1901-1968 used for the period of interest

$T_{\text{Max}}'$ = average daily maximum temperature in the month of, and the month before collections were made

$T_{\text{Min}}'$ = average daily minimum temperature in the month of, and the month before collections were made